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**13. ABSTRACT** ***(Maximum 200 Words)***

The goal of this project is to characterized common molecular mechanism of neuronal toxicity mediated by glutamate and polyglutamine-expanded huntingtin. Our results show that activation of mixed-lineage kinase and its down-stream signaling pathway is involved in neuronal toxicity mediated by glutamate and by the mutated huntingtin. We also observed that PSD-95, a scaffold protein that binds to glutamate receptors links glutamate receptor to mixed lineage kinase to induce neuronal toxicity. Moreover, our results also show that normal huntingtin bind and inhibit mixed lineage kinase while polyglutamine-expanded huntingtin loses this important function and promotes neuronal toxicity induced by glutamate receptors. Our results indicate that an inhibitor for mixed lineage kinase or C-Jun N-terminal kinase may be beneficial for the prevention of neuronal loss in Huntington's disease and perhaps other neurodegenerative diseases and brain damages induced by acute insult.

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## INTRODUCTION

Glutamate-mediated excitotoxicity has been implicated in neuronal loss in many neurodegenerative diseases and brain damages caused by acute insults. It is also a common cause of neuronal death due to battle and non-battle injuries or exposure to various military hazards. Our hypothesis is that a common molecular mechanism may exist for the induction of neuronal toxicity induced by both glutamate and mutated huntingtin. Our goal is to characterize this common signaling mechanism and to define the role of each signaling molecule involved in both glutamate- and mutated huntingtin-mediated neuronal toxicity and to identify potential drug targets for the prevention of neuronal loss in various neurodegenerative diseases and brain damages in acute insults.

## BODY

We have been characterized the interaction of huntingtin with MLK2 (1). We found that normal huntingtin bind to MLK2 and huntingtin's N-terminal proline rich region and the SH3 domain of MLK2 mediates the interaction. Polyglutamine expansion of huntingtin impairs its ability to bind to MLK2, leading to over-activation of MLK2-mediated cellular signaling pathway and neuronal toxicity (1). We have generated kinase dead MLK2 and MKK4 and MKK7 and further demonstrated that co-expression of these dominant negative mutants significantly inhibits JNK activation and neuronal toxicity induced by the mutated huntingtin. We also found that normal huntingtin sequester MLK2 thereby inhibiting the kinase activity and co-expression of normal huntingtin significantly inhibits neuronal toxicity mediated by polyglutamine-expanded huntingtin. These data have been published on Journal Biological Chemistry (see appendices for detailed data reports).

We next investigated the interaction of MLK2 and MLK3 with PSD-95. We found that MLK2 or MLK3 was associated with PSD-95 in HN33 cells and in the rat brain. The SH3 domain of PSD-95 mediates its interaction with MLK2 or MLK3. Activation of kainate receptor GluR6 subunits induces JNK activation and neuronal apoptosis, which was significantly attenuated by co-expression of kinase dead MLK2 or SH3 domain-deleted PSD-95. Our data suggest that PSD-95 links GluR6 to MLK2 or MLK3 cascades to induce JNK activation and neuronal toxicity (2). These data have been submitted to Journal Biological Chemistry and accepted for publication and will be printed out in the coming April (see appendices for detailed data reports).

We next examined the interaction of normal huntingtin with PSD-95 and determined the potential physiological and pathological relevance of the interaction. First, we studied the interaction of normal huntingtin with PSD-95. Full-length PSD-95 expressing vector was transiently transfected in 293T cells. 48 hours post-transfection, cell lysates were prepared and co-immunoprecipitation was conducted using 437, an anti-huntingtin N-terminal antibody or a monoclonal antibody specific for PSD-95. The resulting blot was probed with the anti-PSD-95 antibody. As shown in Fig. 1A, PSD-95 was detected in both 437 or PSD-95 (positive control) immunoprecipitates of 293T cell lysates transfected with PSD-95 but not in 437 immunoprecipitates of wild-type or vector-transfected 293T cell lysates. Next, we explored association of huntingtin with PSD-95 in human cortex tissues. Brain lysates were immunoprecipitated with 437 or negative control, protein A sepharose beads alone, 437 pre-immune serum or 437 pre-absorbed with its peptide antigen and the blot was analyzed by an anti-PSD-95 antibody. As shown in Fig. 1B, PSD-95 was only detected in 437 immunoprecipitates but not in any of negative controls. Conversely, huntingtin was also present in PSD-95 immunoprecipitates (Fig. 1C). These data suggest that huntingtin is associated with PSD-95 in human cortex.

PSD-95 is known to bind to the C-terminus of NMDA receptor NR2 subunits and kainate receptor GluR6 subunit (3-4). If huntingtin is associated with PSD-95, it may also assemble complexes with NMDA and GluR6 receptors. Thus, we investigated whether huntingtin is associated with NMDA and kainate receptors. Human cortex lysates were incubated with anti-NR1, NR2A, NR2B, GluR6, or dopamine D4 receptors, 437 or 437 pre-immune serum and the blot was probed with 437. As shown in Fig. 1C, huntingtin was found in anti-NR1, NR2A, NR2B, GluR6, and 437 immunoprecipitates but not in anti-dopamine D4 receptor and 437 pre-immune serum. These data suggest that huntingtin assemble complexes with NMDA and GluR6 receptors in human cortex.

Our studies and others have shown that huntingtin binds to type II SH3 domain (5-6). PSD-95 has a type II SH3 domain; therefore, we examined whether normal huntingtin binds to the SH3 domain of PSD-95. GST alone or different PSD-95 GST fusion proteins were prepared and 1-2 μg of these PSD-95 GST fusion proteins were incubated with wild-type 293T cell lysates. As shown in Fig. 2, huntingtin only binds to SH3 or SH3-GK domain but not GST alone, GK domain or any of three PDZ domains of PSD-95. These data indicate that the SH3 domain of PSD-95 mediates its binding to huntingtin.

We next determined whether expansion of the polyglutamine repeat in huntingtin would alter its interaction with PSD-95 and NMDA or kainate receptors. We have shown that the N-terminal proline-rich region adjacent to the polyglutamine repeat binds to the SH3 domain of MLK2 (1). Thus, we examined the binding of PSD-95 to huntingtin's N-terminus containing either normal or expanded polyglutamine stretch. Huntingtin's N-terminus GST fusion proteins containing 16 or 56 polyglutamine repeats were generated and purified. These GST fusion proteins were incubated with 293T cell lysates expressing full-length PSD-95. As shown in Fig. 3A, PSD-95 binds to huntingtin's N-terminus containing 16 polyglutamine repeats. Because the proline region is the only SH3 domain-binding site in this small N-terminal segment of huntingtin, these data suggest that the N-terminal proline region of huntingtin mediates its interaction with PSD-95. The amount of PSD-95 bound to huntingtin's N-terminus with 56 polyglutamine repeats was significantly reduced, about 70% less than that associated with the N-terminus of normal huntingtin (Fig. 3A). This data indicate that expansion of the polyglutamine repeat inhibits its ability to bind to the SH3 domain of PSD-95.

Next, we explored the change of association of normal and mutated huntingtin with PSD-95 in the brains of HD patients. Lysates of human cortex tissues from two normal subjects and two mid-age onset HD patients were prepared and these lysates were incubated with 437. The blots were analyzed by an anti-PSD-95 antibody. As shown in Fig. 3B, huntingtin is associated with PSD-95 in cortex tissues of both normal subjects and HD patients. However, the association of huntingtin with PSD-95 in cortex tissues of HD patients is significantly weaker than that in cortex tissues of normal subjects. Interestingly, the amount of PSD-95 associated with huntingtin in HD patients' cortex tissues is about 80% lower than that in cortex tissues of normal subjects, not 50% lower as one would expect given the heterozygous nature of these HD patients. Conversely, these brain lysates were incubated with anti-PSD-95 and the blot was probed with 437. Consistently, normal huntingtin in cortex tissues of HD patients still interacts with PSD-95 but the amount of huntingtin proteins associated with PSD-95 is well below 50% while polyglutamine-expanded huntingtin was not present in anti-PSD-95 immunoprecipitates (Fig. 3C). These data suggest that polyglutamine-expanded huntingtin in the brain of HD patients may be no longer associated with PSD-95 and normal huntingtin in these patients may be re-distributed. We examined three other normal subjects and HD patients with different post-mortem times and obtained similar results (data not shown). Thus, the difference of the extent of the association of huntingtin with PSD-95 between normal

subjects and HD patients is not due to the difference of post-mortem time but reflects an alteration of huntingtin to interact with PSD-95 in HD patients.

We next investigated the physiological relevancy of the association of huntingtin with PSD-95. PSD-95 is known to regulate NMDA receptor- or GluR6-operated channels by clustering these receptors at post-synaptic membranes. Our hypothesis is that normal huntingtin modulates these receptor-operated channels by binding and sequestering PSD-95 thereby regulating the clustering of these receptors. Because the ability of huntingtin to bind to PSD-95 is severely impaired upon polyglutamine expansion, more PSD-95 proteins may be available to cluster NMDA or GluR6 receptors leading to over-activation or sensitization of these receptors and excitotoxicity. If our hypothesis is correct, expression of polyglutamine-expanded huntingtin may enhance, while over-expression of normal huntingtin may inhibit neuronal toxicity mediated by NMDA or GluR6 receptors. In our previous studies, we observed that over-expression of polyglutamine-expanded huntingtin in HN33 cells induces apoptotic cell death (1). According to Western blot analysis, HN33 cells express both NR1 and NR2A receptors. To test our hypothesis, we examined whether expression of the mutated huntingtin may induce activation of NMDA receptors in HN33 cells. Full-length huntingtin containing 16 or 48 polyglutamine repeats (pFL16HD or pFL48HD) was transiently expressed in HN33 cells and glutamate (1 mM) was included in the transfection medium. 24 or 48 hours post-transfection, cells were fixed and TUNEL staining, which detects apoptotic cells, was conducted. TUNEL negative cells (living cells) were counted. In consistent with our previous reports, HN33 cells transfected with pFL48HD started to undergo apoptosis at ~24 hours post-transfection (Fig. 4). At 48 hours, ~60% of HN33 cells were apoptotic (Fig. 4). Treatment of wild-type HN33 cells or cells transfected with pFL16HD with glutamate did not alter cell viability (Fig. 4). Treatment of HN33 cells transfected with pFL48HD with glutamate significantly promoted neuronal toxicity. At 24 hours post-transfection, ~35-40% of HN33 cells were apoptotic when glutamate was added into the transfection medium, comparing with 10-15% of apoptotic cells without glutamate (Fig. 4). At 48 hours, 80% of HN33 cells were apoptotic when treated with glutamate comparing with ~60% of apoptotic cells (Fig. 4). These data suggest that expression of polyglutamine-expanded huntingtin renders sensitization and activation of glutamate receptors, leading to neuronal toxicity.

**Figure legends**:

**Fig. 1**. The association of normal huntingtin with PSD-95. All data presented are from a typical experiment, which has been repeated twice with similar results. **1A**. Detection of PSD-95 in huntingtin immunoprecipitates: Lysates of wild-type 293T cells (W.437) or transfected with the vector alone (V.437) or full-length PSD-95 were incubated with 437 (95.437) or an anti-PSD-95 antibody (95. PSD) and the blot was analyzed by an anti-PSD-95 antibody. **1B:** Association of huntingtin with PSD-95 in human cortex. Human cortex lysates were incubated with 437, protein A beads alone (Beads alone), the 437 pre-immune serum (437.Pre.) or 437 pre-absorbed with its peptide-antigen (437. P.ab) and the blot was analyzed by an anti-PSD-95 antibody. **1C**. Association of huntingtin with Glutamate receptors. Human cortex lysates were incubated with an antibody specific for NR1, NR2A, NR2B, GluR6, PSD-95 or dopamine D4 receptor (D4R) or 437 or 437 pre-immune serum and the blot was analyzed by 437.

**Fig. 2. The SH3 domain of PSD-95 mediates its binding to huntingtin**. 293T cell lysates were incubated with ~2 μg of GST alone or different PSD-95 GST fusion proteins and the blot was analyzed with 437. SH3, SH3-GK, GK, PDZ1, PDZ2 or PDZ3 indicates different PSD-95 GST fusion proteins containing SH3, SH3-GK, GK, PDZ1 or 2 or 3 domain. 437Ip, immunoprecipitation with 437. The data represents a typical experiment that has been repeated three times with similar results.

**Fig. 3.** The polyglutamine expansion inhibits huntingtin to bind to PSD-95. All data presented are from a typical experiment that has been repeated at least three times with similar results. **3A.** The polyglutamine expansion inhibits huntingtin's N-terminus to bind to PSD-95: 293T cell lysates transfected with full-length PSD-95 were incubated with GST alone or huntingtin's N-terminus GST fusion proteins containing 16 (16CAG) or 56 (56CAG) polyglutamine repeats and the blot was analyzed by an anti-PSD-95 antibody. **3B & 3C**. Decreased association of huntingtin with PSD-95 in HD cortex. Human cortex lysates from two normal subjects and two HD patients were precipitated with 437 (3B) or an-PSD95 (3C) and the blot was analyzed by an anti-PSD-95 (3B) or 437 (3C). N1IP or N2IP, immunoprecipitation with 437 or anti-PSD-95 of cortex lysates from normal subject 1 or 2; HD1IP or HD2IP, immunoprecipitation with 437 or anti-PSD-95 of cortex lysates from HD patient 1 or 2; N1Ly., N2Ly. or HD1Ly., HD2Ly. cortex lysates from normal subject 1 or 2 or HD patient 1 or 2.

**Fig. 4**. Expression of polyglutamine-expanded huntingtin promotes neuronal toxicity mediated by NMDA receptors. HN33 cells were transfected with pFL48HD in the $Mg^{++}$-free medium. Glutamate (1 mM) was added in the transfection medium. Cells were fixed at the time of post-transfection as indicated in the figure (4A) or 48 hours (4B) and TUNEL stain was conducted and TUNEL negative cells were counted. The number of TUNEL negative cells in the control (transfected with pcDNA1) was designated as 100%. Data presented are the average of three or four independent experiments.

Fig. 1A

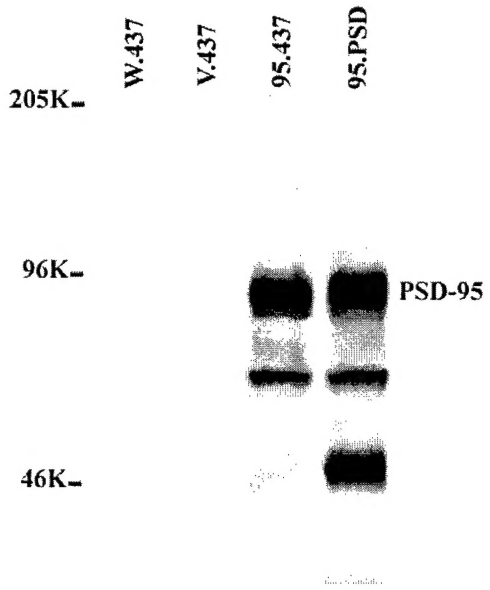
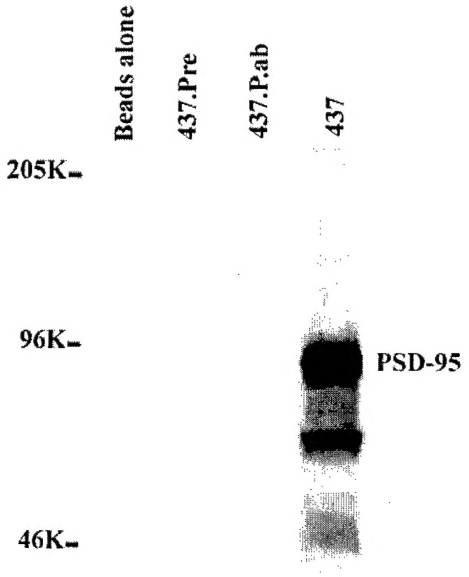



Fig. 1B

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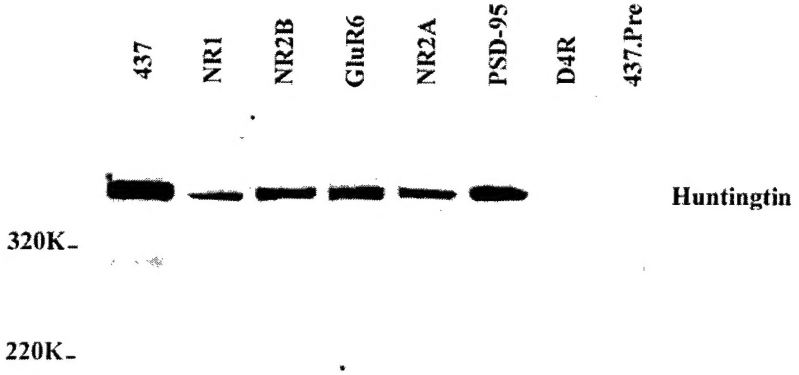


Fig. 2

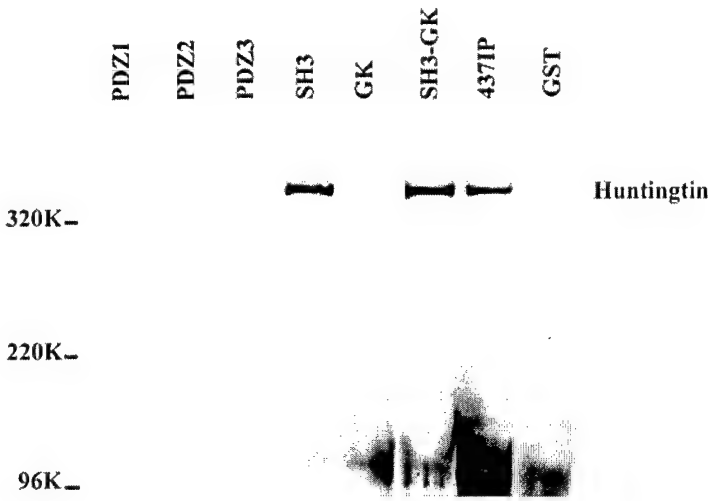



Fig. 3A

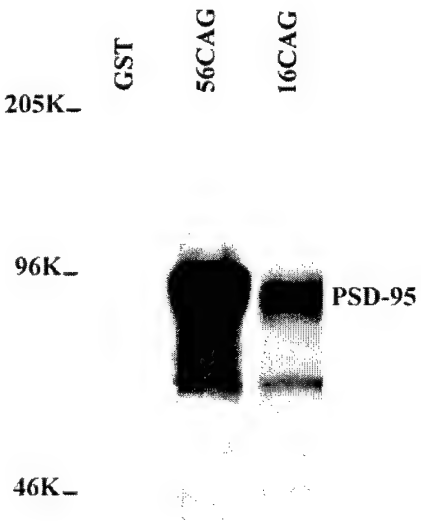



Fig. 3B

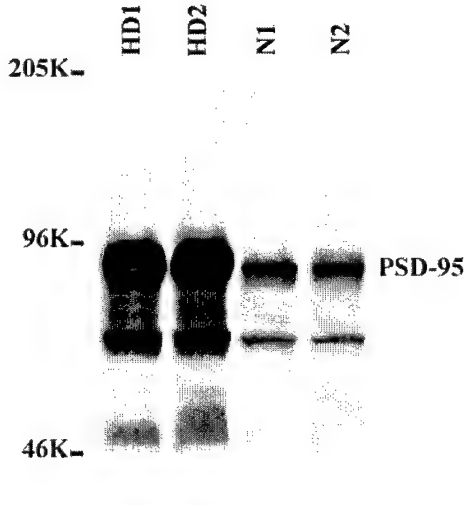


Fig. 3C

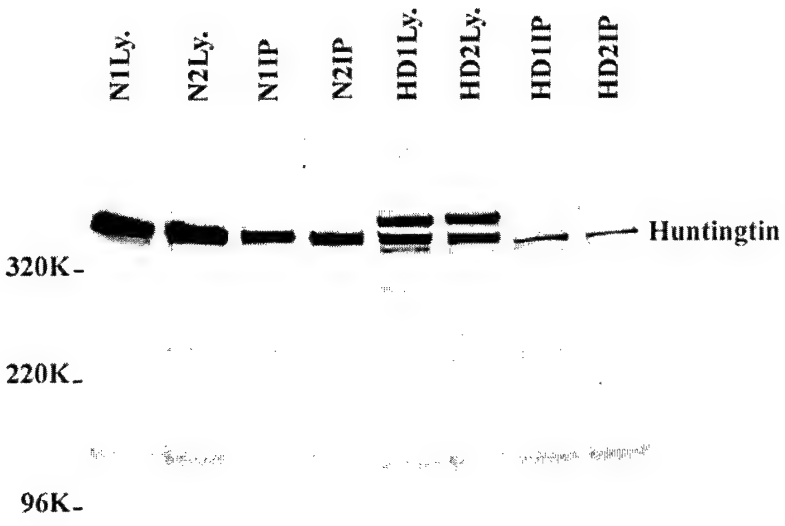
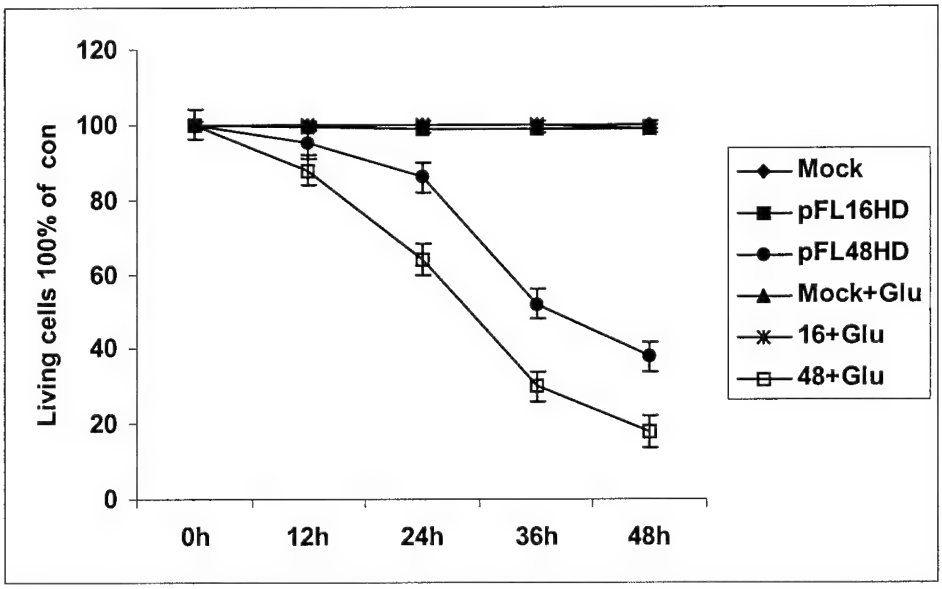

N1Ly.
N2Ly.
N1IP
N2IP
HD1Ly.
HD2Ly.
HD1IP
HD2IP
Huntingtin
320K-
220K-
96K-

Fig. 4


Living cells 100% of con
120
100
80
60
40
20
0
0h
12h
24h
36h
48h
Mock
pFL16HD
pFL48HD
Mock+Glu
16+Glu
48+Glu

## KEY RESEARCH ACCOMPLISHMENTS

1. Normal huntingtin binds and sequester MLK2 thereby inhibiting activation of the kinase and the kinase-mediated neuronal toxicity.

2. Polyglutamine expansion inhibits huntingtin to bind to the SH3 domain of MLK2 and impairs its ability to inhibit MLK2-mediated cellular signaling cascades in neuronal cells leading to over-activation of JNK and neuronal apoptosis.

3. Co-expression of normal huntingtin inhibits JNK activation and neuronal toxicity mediated by polyglutamine-expanded huntingtin and by MLK2.

4. MLK2 or MLK3 binds to PSD-95.

5. PSD-links GluR6 to MLK, which induces neuronal toxicity.

6. Normal huntingtin binds and sequester PSD-95.

7. Polyglutamine-expanded huntingtin inhibits normal huntingtin to bind to PSD-95 and promotes neuronal toxicity induced by glutamate receptors

## REPORTABLE OUTCOMES

1. **Publications**:

1). Liu, Y.F., Dorow, D. and Marshall, J. (2000) *J. Biol. Chem.* **275**, 19035-19040.

2). Savinainen, A. Garcia, E.P., Dorow, D., Marshall J., and Ya Fang Liu (2001) *J. Biol. Chem* (in press).

**2. Abstracts:**

1). Ya Fang Liu (2000) Activation of MLK is involved in neuronal toxicity induced by GluR6 receptors. Neurobiology Conference, Turin, Italy

2) Ya Fang Liu and Ying Sun (2000) Association of huntingtin with synaptic associated proteins. Society for Neuroscience, New Orleans, LA

**3. Degree:**

Ying Sun, Master degree in Pharmaceutical Sciences at Northeastern University, 2000

**4. Employment:**

The following people have been received salary support from the current project budget:

| | |
|---|---|
| Ya Fang Liu | Principal Investigator |
| Ying Sun, | Research Associate |
| Anneli Savinainen, | Research Associate |

## CONCLUSIONS

Our data show that normal huntingtin binds and inhibits glutamate receptor-mediated toxicity via binding and sequestering MLK and PSD-95 while polyglutamine-expansion impairs this important biological function of huntingtin and the mutated huntingtin promotes neuronal toxicity mediated by glutamate. Before our data were presented, it wildly accepted in the Huntington's disease (HD) field that gain-of function causes neuronal loss in HD. Our studies first demonstrate that loss of biological function of huntingtin may play a critical role in the pathogenesis of HD and normal huntingtin is capable to inhibit neuronal toxicity induced by the mutated huntingtin. The data that normal huntingtin inhibits the mutated huntingtin-mediated neuronal toxicity have been recently reproduced by other group using HD transgenic mice (7). Now, the HD field starts to accept that loss of function is more important in the pathogenesis of HD (8). Therefore, our results provide the initial evidence for this theory; undoubtedly, we have made a very significant contribution in understanding of the pathogenesis of HD.

Our studies demonstrate that PSD-95 links kainate receptors to MLK and over-activation of the MLK-JNK is involved in excitotoxicity mediated by the receptors. Activation of kainate receptors has been implicated in stroke. Therefore, an MLK or JNK inhibitor may be helpful for the prevention of neuronal loss in acute insults.

## REFERENCES:


1. Liu, Y.F., Dorow, D. and Marshall, J. (2000) *J. Biol. Chem.* **275**, 19035-19040.
2. Savinainen, A. Garcia, E.P., Dorow, D., Marshall J. and Ya Fang Liu (2001) *J. Biol. Chem* (in press).
3. Kim, E., Cho, K.O., Rothschild, A., Sheng, M. (1996) *Neuron*, **17**, 103-113.
4. Garcia, E.P., Metha, S., Blair, L.A., Wells, D.G., Shang, J., Fukushima, T., Fallon, J.R., Garmer, C.C. and Marshall, J. (1998) *Neuron*, **21**, 727-736.
5. Liu, Y.F., Deth, C.R. and Devys, D. (1997) *J. Biol. Chem.* **272**, 8121-8124.
6. Sitter, A., Wälter, S., Wedemeyer, N., Hasenbank, R., Scherzinger, E., Eickhoff, H., Bates, G.P., Lehrach, H., and Wanker, E.E.(1998) *Mol. Cell* **2**, 427-436 .
7. Leavitt, B.R., Guttman, J.A., Hodgson, J.G., Kimel, G.H., Singaraja, R., Vogal, A.W. and Hayden, M.R. (20001) *Am J. Hum. Genet.* **68**, 313-324.

8. Cattaneo, E., Rigamonti, D., Goffredo, D., Zuccato, C., Squitieri, F., Sipione, S (2001) *Trends Neurosci.* **24**, 182-188.

# APPENDICES FOR Dr. Ya Fang Liu

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# Activation of MLK2-mediated Signaling Cascades by Polyglutamine-expanded Huntingtin*

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**We previously reported that expression of polyglutamine-expanded huntingtin induces apoptosis via c-Jun amino-terminal kinase (JNK) activation in HN33 cells (Liu, Y. F. (1998) *J. Biol. Chem.* 273, 28873–28822). Extending this study, we now demonstrate a role of mixed-lineage kinase 2 (MLK2), a JNK activator, in polyglutamine-expanded huntingtin-mediated neuronal toxicity. We find that normal huntingtin interacts with MLK2, whereas the polyglutamine expansion interferes with this interaction. Similar to the expression of polyglutamine-expanded huntingtin, expression of MLK2 also induces JNK activation and apoptosis in HN33 cells. Co-expression of dominant negative MLK2 significantly attenuates neuronal apoptosis induced by the mutated huntingtin. Furthermore, over-expression of the N terminus of normal huntingtin partially rescues the neuronal toxicity induced by MLK2. Our results suggest that activation of MLK2-mediated signaling cascades may be partially involved in neuronal death induced by polyglutamine-expanded huntingtin.**

Huntington's disease (HD)[1] is a neurodegenerative disorder with dominant inheritance (2). The disease is characterized by choreiform movement, mental impairment, and cognitive symptoms (3, 4). The HD gene encodes a 350-kDa protein designated as huntingtin (2), which is ubiquitously expressed with the highest levels being found in the brain, lung, and testes (5, 6). Immunocytochemistry reveals that in neurons huntingtin is a cytoplasmic protein found in cell bodies, dendrites, and also in nerve terminals, where huntingtin is associated with synaptic vesicles and microtubule complexes (6, 7). The defect in the HD gene causes an expansion of a polyglutamine stretch near the N terminus of huntingtin, and the length of the polyglutamine repeat is correlated with the age of onset and the severity of the disease (8). To date, the normal function of huntingtin remains to be determined, and the molecular mechanism underlying neuronal death in HD is poorly understood.

In previous studies, we found that expression of polyglutamine-expanded huntingtin caused neuronal apoptosis via activation of JNKs in HN33 cells, a hippocampal neuron-derived cell line (1). The aim of the present study was to investigate the molecular mechanism by which polyglutamine-expanded huntingtin activates JNKs and induces neuronal apoptosis. Huntingtin contains multiple proline-rich motifs that may bind to both SH3 and WW domain-containing proteins (9). Interestingly, the N-terminal proline-rich region, which is about 40 amino acids long, is adjacent to the polyglutamine stretch. This proline-rich region has been shown to bind to both SH3 and WW domain-containing proteins (10–12). MLK2 is a member of the mixed-lineage kinase family whose kinase domain shows structural features of both tyrosine-specific and serine/threonine-specific protein kinases (13). MLK2 possesses an SH3 domain that is homologous to the SH3 domains of Grb2 (13). MLK2 is predominantly expressed in the brain (13), and it has been reported that MLK2 can directly bind and mediate activation of MKK7 and SEK1, which in turn induces JNK activation (14–19). At moderate expression levels, MLK2 appears to selectively activate JNKs and has little effect on other mitogen-activated protein kinases (14–16). Thus, MLK2 is a potential candidate for the involvement in JNK activation and neuronal toxicity induced by polyglutamine-expanded huntingtin. The current study was undertaken to investigate the role of MLK2 in mutated huntingtin-mediated neuronal toxicity. Our results suggest that huntingtin binds to the SH3 domain of MLK2 and the polyglutamine expansion interferes with its binding to the kinase. Activation of MLK2-mediated signal transduction pathways may be involved in initiating neuronal death in HD.

## MATERIALS AND METHODS

*Cell Culture and Transient Transfection*—HN33 cells, an immortalized rat hippocampal neuronal cell line (1), and 293T cells, human embryonic kidney cells expressing SV40 large T antigen, were maintained in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% fetal bovine serum on 10-cm plates. HN33 or 293T cells at 50 to 60% confluence were washed once with serum-free medium prior to transfection. Transfection was performed using Lipofectin (Life Technologies, Inc.) according to manufacturer instructions. 10–50 μg of plasmid with 10–20 μl of Lipofectin/10-cm plate was used in transfection experiments.

*Western Blotting and Immunoprecipitation*—48–72 h after transfection, 293T cells were harvested and lysed in 1% Nonidet P-40 lysis buffer, and co-immunoprecipitation experiments were conducted as described previously using an anti-huntingtin's N terminus antibody 437 (10) or anti-c-Myc-tagged antibody 9E10 (Santa Cruz). Human brain tissues were obtained from Dr. J.-P. Vonsattel or Human Brain Bank at McLean Hospital, Boston, MA with institutional review board approval. Post-mortem time was between 10–12 h. The diagnosis of HD was confirmed with neuropathological and genetic phenotype analysis. Human cortex tissues from normal subjects or HD patients were ho-

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This work is dedicated to Dr. R.-Y. Zhong.

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[1] The abbreviations used are: HD, Huntington's disease; MLK2, mixed lineage kinase 2; JNK(s), c-Jun amino-terminal kinase(s); MKK7, mitogen-activated protein kinase 7; SEK1, stress-signaling kinase 1; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; TUNEL, Tdt-mediated dUTP-biotin nick end labeling; IP, immunoprecipitation.

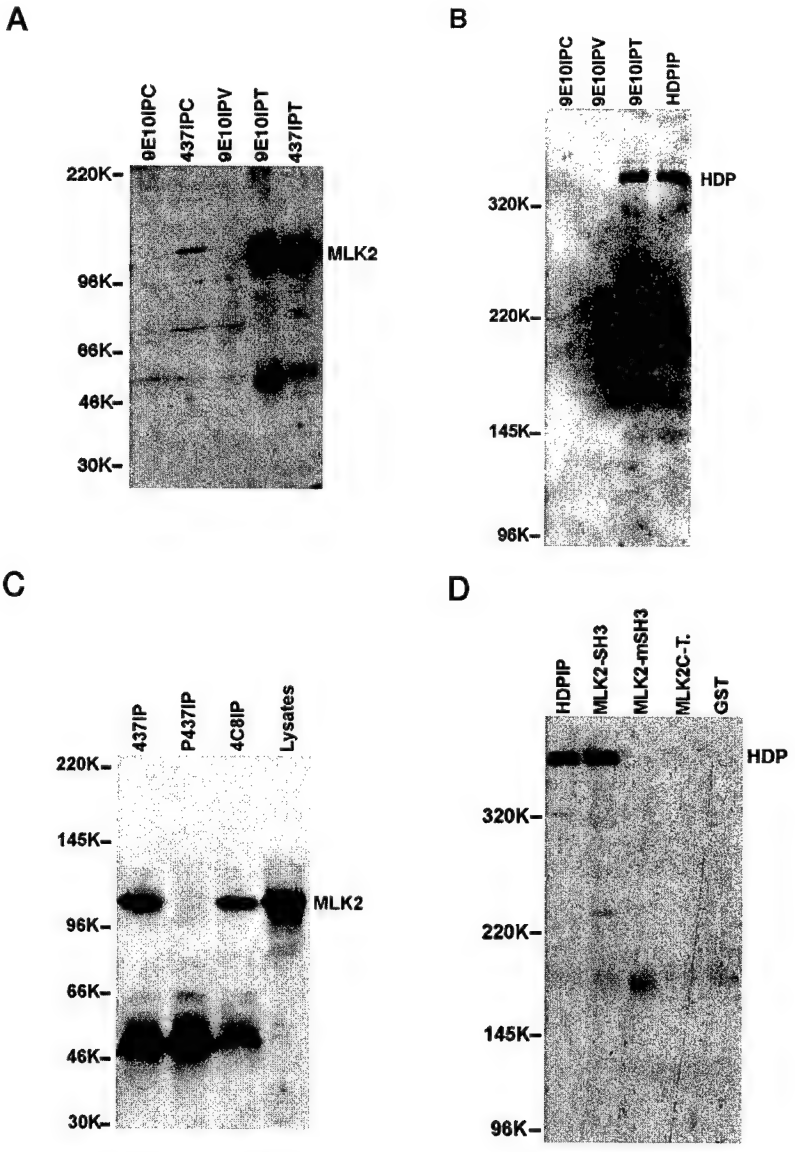


FIG. 1. **The association of normal huntingtin with MLK2.** All data presented are from a typical experiment that has been repeated twice with similar results. *A*, detection of MLK2 in huntingtin immunoprecipitates was as follows: 293T cell lysates of wild-type (*437IPC* and *9E10IPC*) or transfected with pRK5 vector alone (*9E10IPV*) or c-Myc-tagged MLK2 (*437IPT* and *9E10IPT*) were incubated with 9E10 or 437, and the blot was analyzed by 9E10. *B*, detection of huntingtin in MLK2 immunoprecipitates was as follows: 293T cell lysates of wild-type (*9E10IPC*) or transfected with vector alone (*9E10IPV*) or c-Myc-tagged MLK2 (*9E10IPT*) were incubated with 9E10, and the blot was analyzed by 4C8 (3). 437 immunoprecipitation (*HDPIP*) was a positive control. *HDP*, huntingtin. *C*, detection of endogenously expressed MLK2 in huntingtin immunoprecipitates was as follows: HN33 cell lysates were incubated with 437 or 4C8, and the blot was analyzed by an anti-MLK2 antibody characterized previously (36). *437IP*, IP with 437; *P437IP*, IP with peptide-antigen preabsorbed 437; *4C8IP*, IP with 4C8. *D*, the SH3 domain of MLK2 mediated its interaction with huntingtin as follows: GST alone or GST fusion proteins of MLK2-SH3 domain (*MLK2-SH3*), MLK2-C terminus (*MLK2C-T.*), or MLK2-deficient SH3 domain (*MLK2-mSH3*) were incubated with 293T cell lysates, and the blot was analyzed by 437.

mogenized in detergent-free lysis buffer, and Nonidet P-40 was added to a final concentration of 1%. The mixture was then incubated at 4 °C with constant shaking for 1–2 h, and insoluble fractions were removed by centrifugation.

*Purification of GST Fusion Proteins and in Vitro Binding Assay*—Huntingtin N-terminal GST fusion protein constructs were generated by subcloning of a cDNA fragment encoding the first three exons of the HD gene containing 16 or 56 CAG repeats into pGEX2T. Construct for MLK2 C terminus (amino acids 407–953) GST fusion proteins was a generous gift of Dr. Alan Hall (University College London, London, UK) (14). The MLK2 SH3 domain cDNA fragment was amplified by reverse transcription-PCR and inserted into pGEX4T1. To generate MLK2 SH3-deficient GST fusion proteins, substitution of the first tryptophan at position 58 of the highly conserved tryptophan doublet of the SH3 domain to lysine was achieved by overlapping extension using PCR with mutated oligonucleotides. Such a substitution eliminates the ability of SH3 domains to bind to proline-rich ligands (10). Expression and purification of different MLK2 or huntingtin GST fusion proteins was performed as described previously, and ~0.1 μg of GST fusion protein was used for *in vitro* binding studies.

*Subcloning and Mutagenesis*—The full-length c-Myc-tagged MLK2 was a gift from Dr. Alan Hall (14). A kinase-dead version of MLK2 was generated by introduction of an Ala-Gly point mutation at position 651 (codon AAG to GAG) by overlapping PCR extension with mutated oligonucleotides, to result in an amino acid substitution of Lys to Glu at the ATP binding loop of the kinase domain. To generate an expression vector for huntingtin's N terminus with 16 CAG repeats, a cDNA fragment was excised from pFL16HD with *Not*I and *Sph*I and subcloned into pcDNA 1.1 (Invitrogen).

*JNK and TUNEL Assays*—16 h after transfection, HN33 cells were lysed with 1% Triton X-100 lysis buffer (1). JNK was assayed as described previously (1). For TUNEL assay, HN33 cells were plated on a slide culture chamber. After transfection, cells were fixed at the time indicated in the figures, and TUNEL staining was performed as described previously (1). Most apoptotic HN33 cells were detached from the slides, and TUNEL stain was performed on remaining attached cells. TUNEL stain-negative cells (living cells) were counted in the 20× power field in four different locations on the slides and ~600–800 cells were counted in the control (1).

## RESULTS

293T cells, which are rich in huntingtin (10), were utilized to study the interaction of huntingtin with MLK2. c-Myc-tagged MLK2 was transiently expressed in 293T cells, and cell lysates were immunoprecipitated with 437, an anti-huntingtin N-terminal antibody (10), and the resulting blot was probed with 9E10, an anti-c-Myc-tagged monoclonal antibody. MLK2 was detected in 437 and 9E10 immunoprecipitates of lysates transfected with c-Myc-tagged MLK2 but not in the negative controls, 9E10 or 437 immunoprecipitates of wild-type or vector-transfected 293T cell lysates (Fig. 1*A*). Conversely, we also found that huntingtin protein was present in 9E10 immunoprecipitates of c-Myc-tagged MLK2-transfected 293T cell lysates but not wild-type or vector-transfected lysates (Fig. 1*B*).

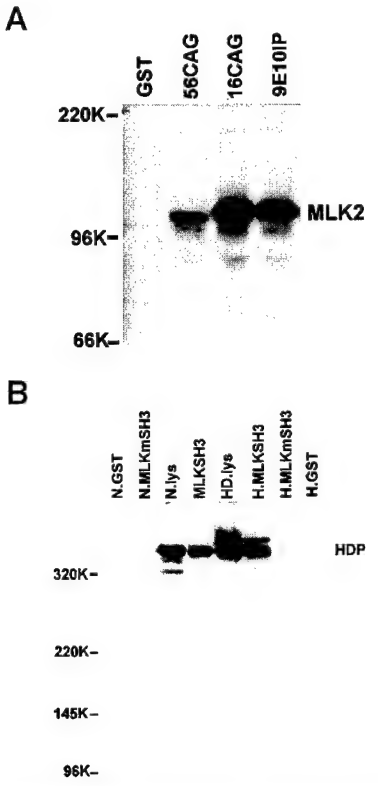


FIG. 2. **The polyglutamine expansion interferes with huntingtin to bind to the SH3 domain of MLK2.** All data presented are from a typical experiment that has been repeated at least three times with similar results. *A*, the polyglutamine expansion inhibits huntingtin's N terminus to bind to MLK2; 293T cell lysates transfected with c-Myc-tagged MLK2 were incubated with GST alone or huntingtin's N terminus GST fusion proteins containing 16 (*16CAG*) or 56 (*56CAG*) polyglutamine repeats, and the blot was analyzed by 9E10 (*9E10IP*). *B*, the ability of the mutated huntingtin to bind to the SH3 domain of MLK2 is impaired. Human cortex lysates from a normal subject and an HD patient were incubated with GST alone or GST fusion proteins of MLK2-SH3 domain (*MLKSH3*) or MLK2-deficient SH3 domain (*MLKmSH3*), and the blot was analyzed by 437. *N. lys* or *N.*, normal human cortex lysates; *HD. lys* or *H.*, HD patient's cortex lysates.

These data show that MLK2 is associated with huntingtin in intact cells.

Next, we examined the interaction of MLK2 with huntingtin in a hippocampal neuronal cell line, HN33 cells in which we found that expression of polyglutamine-expanded huntingtin induced apoptosis (1). MLK2 is richly expressed in HN33 cells, and the amount of MLK2 proteins in the cell is similar to that of 293T cells over-expressing c-Myc-tagged MLK2 (Fig. 1*C*). HN33 cell lysates were immunoprecipitated with 437 or 4C8, a well characterized anti-huntingtin monoclonal antibody (5), and the resulting blot was probed with a specific anti-MLK2 antibody that has been characterized previously (20). As shown in Fig. 2*B*, both 437 and 4C8 were able to precipitate MLK2 from HN33 cell lysates, whereas the peptide-antigen pre-absorbed 437 failed to do so. These data provide further evidence that normal huntingtin is associated with MLK2 in neuronal cells.

To determine whether the SH3 domain of MLK2 mediates its interaction with huntingtin, we investigated the binding of huntingtin to a GST MLK2-SH3 domain fusion protein, whereas a GST MLK2 C terminus fusion protein, which lacks the SH3 domain, served as a negative control. As shown in Fig. 1*D*, huntingtin binds to the MLK2 SH3 domain but not GST alone or the MLK2 C terminus. To verify whether the SH3 domain of MLK2 mediates its interaction with huntingtin, we generated a MLK2-SH3 domain-deficient GST fusion protein by substitution of the first tryptophan of the highly conserved tryptophan doublet of the MLK2 SH3 domain to lysine. Mutation of the tryptophan doublet of the SH3 domain is known to eliminate its ability to bind to proline-rich ligands (10). Huntingtin failed to bind to MLK2-SH3 domain-deficient GST fusion proteins (Fig. 1*D*). These data show that the SH3 domain of MLK2 mediates its interaction with normal huntingtin.

We next determined whether expansion of the polyglutamine repeat in huntingtin would alter its interaction with MLK2. The N-terminal proline-rich region adjacent to the polyglutamine repeat has been reported to bind to SH3 domain-containing proteins (10–11). Thus, we examined the binding of MLK2 to huntingtin's N terminus containing either a normal or expanded polyglutamine stretch. GST fusion proteins of huntingtin's N terminus containing 16 or 56 polyglutamine repeats were generated and purified. These GST fusion proteins were utilized as a template to examine whether huntingtin's N terminus is responsible for its interaction with MLK2 and how expansion of the polyglutamine repeat affects this interaction. As shown in Fig. 2*A*, MLK2 binds to huntingtin's N terminus containing 16 polyglutamine repeats. Because the N-terminal proline region is the only potential SH3 domain binding site in this small N-terminal segment of huntingtin and others have shown that this region mediates huntingtin binding to SH3 domain (11), these data suggest that the N-terminal proline region is involved in huntingtin interaction with MLK2. The amount of c-Myc-tagged MLK2 bound to huntingtin's N terminus with 56 polyglutamine repeats was significantly reduced, about 70% less than that associated with the N terminus of normal huntingtin (Fig. 2*A*). This data indicates that expansion of the polyglutamine repeat may inhibit the ability of huntingtin's N terminus to interact with the SH3 domain of MLK2.

Next, we examined the interaction of MLK2 with huntingtin in the human brain. Because our MLK2 antibody cannot be used for immunoprecipitation, a MLK2 SH3 domain GST fusion protein was used to test the ability of normal and polyglutamine-expanded huntingtin proteins from human brain tissues to bind to MLK2. Lysates of human brain cortex tissues from a normal subject and a mid-age onset HD patient were prepared. Wild-type or mutated MLK2 SH3 domain GST fusion protein was incubated with human brain lysates, and the resulting blot was probed with 437. As shown in Fig. 2*B*, normal huntingtin protein from normal or HD human cortex tissues bound to the wild-type MLK2 SH3 GST fusion protein but not to GST alone or to the MLK2 SH3 domain-deficient GST fusion protein. In contrast, polyglutamine-expanded huntingtin protein from the HD patient brain only weakly bound to the SH3 domain of MLK2 (Fig. 2*B*). These data further support our findings that normal huntingtin binds to the SH3 domain of MLK2 and that the polyglutamine expansion interferes with its ability to interact with the SH3 domain of the kinase.

MLK2 is known to induce JNK activation in Cos-1 cells (14). Therefore, we tested whether expression of MLK2 activates JNKs in HN33 cells. The MLK2 expression vector (pRK5) or vector alone was transiently transfected into HN33 cells. JNKs were precipitated using a GST-c-Jun protein, and an *in vitro* JNK assay was performed. An equal amount of JNK proteins were precipitated in each JNK assay (data not shown). As observed in other neuronal cells (21), a basal level of JNK activity was found in HN33 cells (Fig. 3*A*). MLK2 induced constitutive activation of JNKs in HN33 cells. As shown in Fig. 3*A*, the level of the JNK activity was increased by 8-fold (Fig. 3, *A* and *B*). Because MLK2 mediates JNK activation via phosphorylation and activation of both MKK7 and SEK1 (14–16), we determined whether co-expression of dominant negative

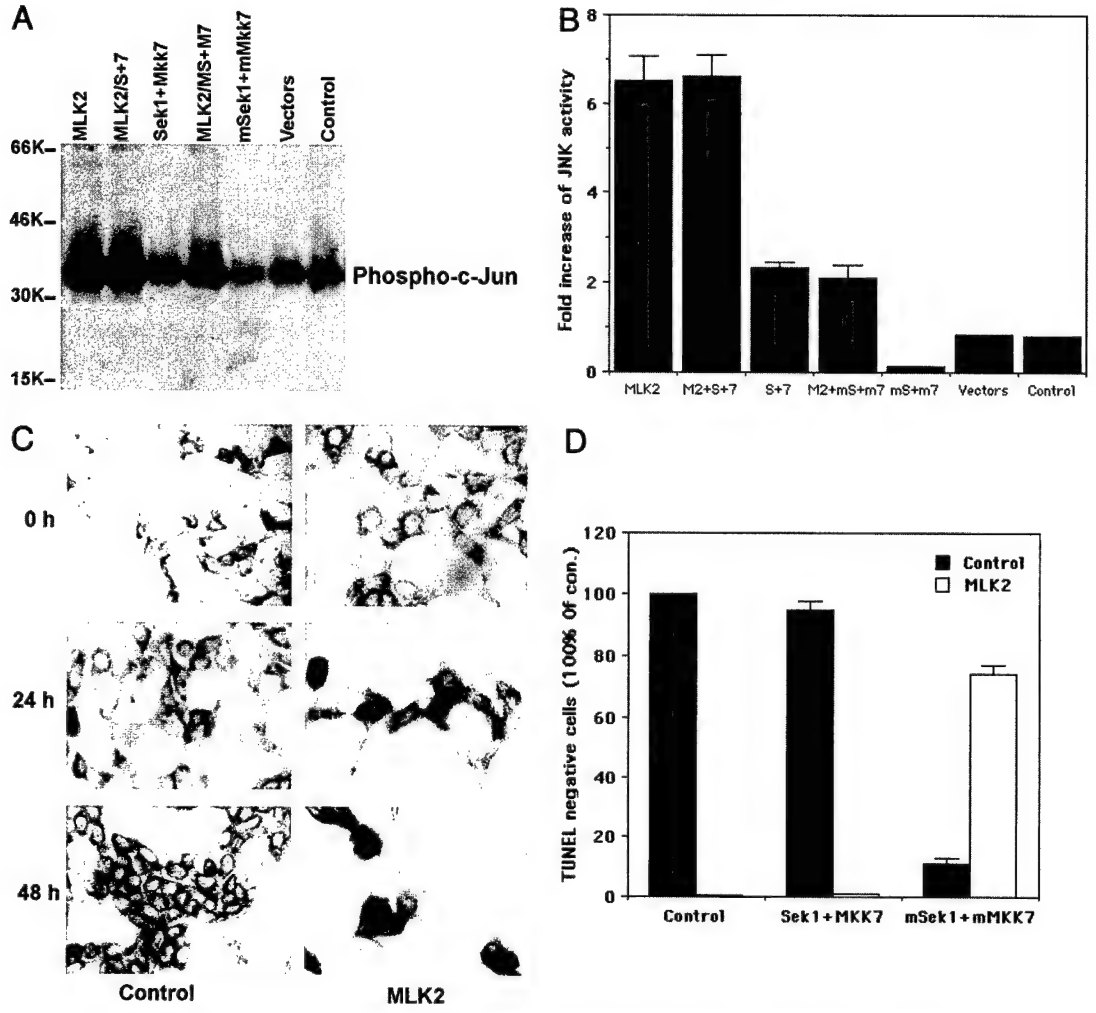


FIG. 3. **Expression of MLK2 in HN33 cells induces JNK activation and neuronal apoptosis.** *A*, expression of MLK2-mediated JNK activation in HN33 cells; HN33 cells were transiently transfected with different plasmids as indicated in the figure. 16 h after transfection, HN33 cells were lysed, and JNK activity was measured as described previously (24). *S*, Sek1; *7*, MKK7, *MS*, dominant negative Sek1 (24); *M7*, dominant negative MKK7; *vectors*, pRK5 and pEBG. *B*, the -fold of the JNK activity induced by MLK2. Increase of JNK activity was determined by analyzing the blots with a densitometer. The values depicted represent the -fold stimulation of JNK activity of HN33 cells transfected with different plasmids as indicated in the figure over the activity of HN33 cells transfected with vector alone. Data are the average of three independent experiments. *C*, expression of MLK2 in HN33 cells induced apoptotic cell death. HN33 cells were transfected with MLK2 expression vector. Following transfection, cells were fixed at the times indicated in the figure, and TUNEL staining was performed as described under "Materials and Methods." Most apoptotic HN33 cells were detached from slides, and TUNEL staining was performed on the remaining cells. Cells showing the retraction of neurites and positive stain in the nucleus were recognized as apoptotic. TUNEL-negative cells (living cells) were counted, and the number of TUNEL-negative cells in the control (transfected with pRK5 + pEBG + pcDNA1) was designated as 100%. Data are the average of three independent experiments. *D*, co-expression of dominant negative MKK7 and SEK1 significantly inhibited MLK2-mediated apoptosis in HN33 cells. HN33 cells were co-transfected with MLK2 expression vector and wild-type or dominant negative SEK1 or MKK7. TUNEL staining was conducted, and TUNEL-negative cells were counted.

MKK7 and SEK1 could block MLK2-mediated JNK activation. As shown in Fig. 3*A*, MLK2-mediated JNK activation was significantly attenuated by co-expression of dominant negative MKK7 and SEK1 but not by co-expression of wild-type MKK7 and SEK1, which had little effect on the JNK activity induced by MLK2 (Fig. 3, *A* and *B*). Co-expression of wild-type MKK7 and SEK1 did not significantly alter basal JNK activity in HN33 cells (Fig. 3, *A* and *B*), whereas co-expression of dominant negative MKK7 and SEK1 inhibited basal JNK activity, which is toxic to HN33 cells (see below Fig. 3*D*).

We have reported that expression of polyglutamine-expanded huntingtin induces neuronal apoptosis by activation of JNKs in HN33 cells (1). Because MLK2 also activates JNKs in HN33 cells, we examined whether expression of MLK2 induced neuronal toxicity in HN33 cells. Vector alone or c-Myc-tagged MLK2 was transiently expressed in HN33 cells and, 24 and 48 h post-transfection, cells were fixed and Tdt-mediated dUTP-biotin nick end labeling (TUNEL) staining, which detects the late stage of apoptosis, was conducted. TUNEL-negative cells (living cells) were counted. Expression of MLK2 induced rapid apoptotic cell death in HN33 cells (Fig. 3*C*). HN33 cells started to undergo apoptosis at ~24 h post-transfection (Fig. 3*C*). At 48 h post-transfection, most HN33 cells were detached from the plate (apoptotic), and remaining attached cells were also apoptotic (Fig. 3*C*). Similar to expression of polyglutamine-expanded huntingtin (1), expression of MLK2 not only led to neuronal death of transfected HN33 cells but also mediated cell toxicity of non-transfected HN33 cells, suggesting that activation of MLK2 may cause the release of cellular diffusible factors that are toxic to neighboring neuronal cells (1).

Next, we investigated whether co-expression of dominant negative MKK7 and SEK1 would inhibit MLK2-mediated neuronal toxicity. Expression of wild-type or dominant negative mutant forms of MKK7 or SEK1 alone did not alter cell viability (1). Co-expression of wild-type SEK1 and MKK7 also did not

generate any cell toxicity (Fig. 3*D*), whereas co-expression of dominant negative SEK1 and MKK7 significantly attenuated neuronal toxicity induced by MLK2. As shown in Fig. 3*D*, ~75% of HN33 cells were rescued upon co-expression of the dominant negative mutant form of MKK7 and SEK1. These data indicate that JNK-mediated neuronal toxicity is induced by MLK2 in HN33 cells. Co-expression of dominant negative MKK7 and SEK1 without MLK2 caused rapid apoptosis in HN33 cells (Fig. 3*D*). Inhibition of basal JNK activity may account for this cell toxicity, because co-expression of dominant negative MKK7 and SEK1 decreased basal JNK activity in HN33 cells (Fig. 3, *A* and *B*), whereas when these two mutated kinases were co-expressed with MLK2 in HN33 cells, JNK activity was double the basal level (Fig. 3, *A* and *B*), and under this condition HN33 cells were viable (Fig. 3*D*). These results suggest that a certain basal level of JNK activity appears to be essential for the survival of HN33 cells, and either over-activation or inhibition of basal JNK activity triggers apoptosis.

To further investigate whether MLK2-mediated signaling cascades are involved in neuronal death induced by polyglutamine-expanded huntingtin, a dominant negative (kinase-dead) form of MLK2, which is known to competitively inhibit the endogenous kinase, was generated. Different full-length huntingtin expression vectors containing 16, 48, or 89 polyglutamine repeats were separately co-transfected with wild-type or the dominant negative form of MLK2 into HN33 cells. As shown in Fig. 4*A*, co-expression of dominant negative MLK2 significantly inhibited neuronal toxicity mediated by polyglutamine-expanded huntingtin in HN33 cells. At 48 h post-transfection of the huntingtin construct containing 48 or 89 CAG repeats, over 75% of HN33 cells remained viable (*i.e.* trypan blue stain-negative) when dominant negative MLK2 is co-expressed, compared with 70–80% of apoptotic cells when the mutated huntingtin was expressed alone (Fig. 4*A*). These data further support a role for MLK2 in the mediation of neuronal toxicity induced by polyglutamine-expanded huntingtin.

Our data show that the N-terminal proline-rich region of huntingtin interacts with MLK2, and the polyglutamine expansion interferes with this interaction. These results suggest that the polyglutamine expansion may lead to an increase in free MLK2 proteins, which are constitutively active and cell toxic (14). If this hypothesis is true, over-expression of the N terminus of normal huntingtin, which binds to free MLK2 proteins, should be able to overcome the neuronal toxicity induced by MLK2 and polyglutamine-expanded huntingtin. We prepared a construct encoding a small region of the normal huntingtin N terminus, containing a 16 polyglutamine repeat and the proline-rich region. As shown in Fig. 4*B*, co-expression of this N-terminal fragment of normal huntingtin significantly attenuated neuronal toxicity mediated by MLK2 and by the mutated huntingtin with 48 polyglutamine repeats. Over 50% of neurons remained viable at 48 h post-transfection, compared with less than 20% of viable cells when the N-terminal fragment was not co-expressed (Fig. 4*B*). These studies support our hypothesis that the polyglutamine expansion in huntingtin may interfere with its interaction with MLK2 thereby leading to an increase of free MLK2 proteins that in turn mediate JNK activation and neuronal apoptosis.

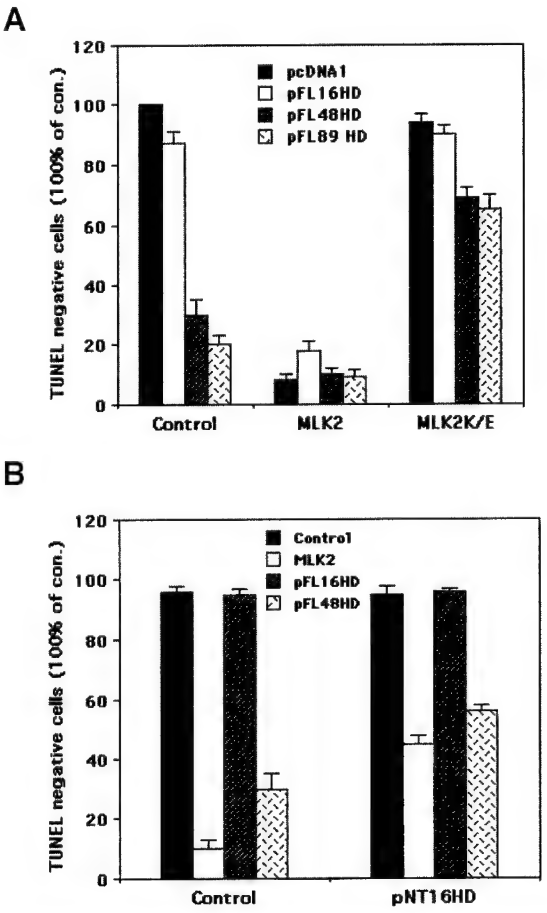


FIG. 4. **MLK2 may be partially involved in polyglutamine-expanded huntingtin-mediated neuronal apoptosis.** HN33 cells were transiently transfected with different plasmids as indicated in the figure. Cells were fixed at 48 h post-transfection, and TUNEL staining was carried out. TUNEL-negative cells were counted, and the number of TUNEL-negative cells transfected with vector (pcDNA1 + pRK5) was designated as 100%. Data are the average of three independent experiments. *A*, co-expression of dominant negative MLK2 significantly attenuated neuronal toxicity mediated by polyglutamine-expanded huntingtin. Full-length huntingtin constructs containing 16, 48, or 89 CAG repeats (1) were transfected alone or co-transfected with wild-type or dominant negative MLK2 (*MLK2K/E*) followed by TUNEL staining. *B*, over-expression of the N terminus of normal huntingtin overcame neuronal toxicity induced by MLK2 and by polyglutamine-expanded huntingtin. The expression vector for the N terminus of normal huntingtin with 16 CAG repeats (*pNT16HD*) was co-transfected with MLK2 or full-length huntingtin with 48 CAG repeats as indicated in the figure. TUNEL-negative cells were counted as described above.

## DISCUSSION

In the present study, we demonstrate that MLK2, an upstream activator of the JNK pathway, is involved in JNK activation and neuronal apoptosis mediated by polyglutamine-expanded huntingtin (1). The polyglutamine expansion decreases the association of huntingtin with MLK2 leading to an increase in unregulated MLK2 proteins that, being constitutively active, cause JNK activation and neuronal toxicity. Co-expression of dominant negative MKK7 and SEK1, the downstream effectors of MLK2, blocks JNK activation and neuronal apoptosis induced by MLK2 and by polyglutamine-expanded huntingtin. Additionally, co-expression of dominant negative MLK2 significantly attenuated JNK activation and neuronal toxicity mediated by the polyglutamine expanded huntingtin. Finally, over-expression of a normal huntingtin N-terminal protein significantly attenuated neuronal toxicity induced by both MLK2 and polyglutamine-expanded huntingtin. These results show that MLK2-mediated cellular signaling cascades may play a significant role in neuronal death induced by polyglutamine-expanded huntingtin in HN33 cells.

Because huntingtin is a ubiquitously expressed protein, whereas the pathology of HD is restricted to the brain, it is likely that huntingtin binds to proteins that are largely found in the brain, and the polyglutamine expansion alters huntingtin's ability to interact with these proteins thereby resulting in activation of neurotoxic pathways. MLK2 being almost exclu-

sively expressed in the brain could provide a partial explanation for why the polyglutamine-expanded huntingtin is selectively toxic to neurons. MLK2 is a strong activator of the JNK pathway that is now known to couple a variety of cell-toxic stimuli, leading to neuronal apoptosis (21, 22). Because MLK2 is a constitutively active kinase, and free MLK2 is the active form (14), any alteration of the amount of bound MLK2 is likely to lead to activation of MLK2-mediated signaling cascades and neuronal toxicity. We show that normal huntingtin proteins from normal or HD human cortex tissues specifically bind to wild-type but not the mutated SH3 domain of MLK2, and under the same conditions, the mutated huntingtin proteins from HD patient cortex tissues only weakly bound to the SH3 domain of MLK2. Moreover, over-expression of the N terminus of normal huntingtin, which binds and decreases free MLK2 proteins in HN33 cells, can significantly inhibit neuronal toxicity induced by MLK2 and by the mutated huntingtin, further supporting the notion that huntingtin's N terminus interacts with the SH3 domain of MLK2, and the ability to bind to the kinase may be impaired upon polyglutamine expansion in huntingtin.

The polyglutamine expansion apparently alters the physical properties of huntingtin. The mobility of the mutated huntingtin on SDS-polyacrylamide gel electrophoresis is clearly decreased (5), and huntingtin's N terminus protein carrying an expanded polyglutamine stretch forms amyloid-like protein aggregates both *in vitro* and *in vivo* (23, 24). Because the N-terminal proline-rich region is adjacent to the polyglutamine stretch, it is possible that the polyglutamine expansion may alter the binding properties of this proline-rich region. Our group previously found that normal huntingtin is associated with epidermal growth factor receptor signaling complexes through binding to the SH3 domains of Grb2 and RasGAP, and this association is regulated by activation of the epidermal growth factor receptor (10). Recently, other groups have also reported that huntingtin binds to Grb2-like SH3 domain-containing proteins, and the N-terminal proline-rich region mediates these interactions (10–11). Our results from the current study are consistent with these reports (10–11). In addition, we show that the ability of this proline-rich region to bind to the SH3 domain of MLK2 is impaired upon expansion of the polyglutamine stretch. The interaction of proline-rich motifs with SH3 domains is not a highly selective event (9). Thus, if the polyglutamine expansion in huntingtin interferes with its interaction with the SH3 domain of MLK2, it may inhibit its association with other SH3 domain-containing proteins. Perhaps the normal function of huntingtin is the modulation of the cellular signaling network by sequestering these SH3 domain-containing signaling proteins. When the ability of huntingtin to interact with SH3 domains is impaired, polyglutamine-expanded huntingtin may be disassociated from the microtubule complex where most SH3 domain-containing proteins are found, leading to the re-arrangement of SH3 domain-containing protein-associated signaling complexes, which may subsequently result in an imbalance of cellular signaling networks and neuronal death. Incidentally, huntingtin's N terminus with an expanded length of polyglutamine repeat forms nuclear inclusions in the brains of HD patients or in cultured cells (25), indicating that some mutated huntingtin proteins are translocated and no longer co-present with MLK2 or other SH3 domain-containing proteins in the cytoplasm.

It is clear now that striatal medium-spiny neurons, which die first in HD, lack endogenous huntingtin. Thus, diffusible neurotoxic factors may play an important role in early neuronal loss in HD. Our previous studies and current results are consistent with this notion; expression of the mutated huntingtin or MLK2 induces not only apoptotic cell death of transfected HN33 cells but also non-transfected cells. Because JNK activation has been reported to mediate free radical production (26), it is possible that polyglutamine-expanded huntingtin may mediate free radical production via activation of the MLK2-JNK pathway. In summary, our current studies show that activation of MLK2-mediated signaling cascades may be partially responsible for neuronal loss in HD, and an inhibitor of MLK2 may be useful for the prevention of neuronal loss in HD.

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REFERENCES

1. Liu, Y. F. (1998) *J. Biol. Chem.* **273,** 28873–28877
2. Huntington's Disease Collaborative Research Group (1993) *Cell* **72,** 971–983
3. Martin, J. B., and Gusella, J. F. (1986) *N. Engl. J. Med.* **315,** 1267–1276
4. Ferrante, R. J., Kowall, N. W., Beal, M. F., Richardson, E. P., Jr., Bird, E. D., and Martin, J. B. (1985) *Science* **230,** 561–563
5. Trottier, Y., Devys, D., Imbert, G., Saudou, F., An, I., Lutz, Y., Weber, C., Agid, Y., Hirsch, E. C., and Mandel, J. L. (1995) *Nat. Genet.* **10,** 104–110
6. Fusco, F. R., Chen, Q., Lamoreaux, W., Figueredo-Cardenas, G., Jiao, Y., Coffiman, J. A., Surmerier, D. J., Honig, M. G., Carlock, L. R., and Reiner, A. (1999) *J. Neurosci.* **19,** 1189–1202
7. Gutekunst, C.-A., Levey, A. J., Hellman, C. J., Whsley, W. L., Yi, H., Nash, R., Rees, H. D., Madden, J. J., and Hersch, S. M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8710–8714
8. Stine, O. C., Pleasant, N., Franz, M. L., Abbott, M. H., Folstein, S. E., and Ross, C. A. (1993) *Hum. Mol. Genet.* **2,** 1547–1549
9. Sudol, M. (1998) *Oncogene* **17,** 1469–1474
10. Liu, Y. F., Deth, R. C., and Devys, D. (1997) *J. Biol. Chem.* **272,** 8121–8124
11. Sitter, A., Wälter, S., Wedemeyer, N., Hasenbank, R., Scherzinger, E., Eickhoff, H., Bates, G. P., Lehrach, H., and Wanker, E. E.(1998) *Mol. Cell* **2,** 427–436
12. Faber, P. W., Barnes, G. T., Srinidhi, J., Chen, J., Gusella, J. F., and MacDonald, M. E. (1998) *Hum. Mol. Genet.* **7,** 1463–1474
13. Dorow, D. S., Devereux, L., Tu, G.-F., Price, G., Nicholl, J. K., Sutherland, G. R., and Simpson, R. J. (1995) *Eur. J. Biochem.* **234,** 492–500
14. Nagata, K. Puls, A., Futter, C., Aspenstrom, P., Schaefer, E., Nakata, T., Hirokawa, N., and Hall, A. (1998) *EMBO J.* **17,** 149–158
15. Hirai, S., Katoh, M., Terada, M., Kyriakis, J. M., Zon, L. I., Rana, A., Avruch, J., and Ohno, S. (1997) *J. Biol. Chem.* **272,** 15167–15173
16. Hirai, S., Noda, K., Moriguchi, T., Nishida, E, Yamashita, A., Deyama, T., Fukuyama, K., and Ohno, S. (1998) *J. Biol. Chem.* **273,** 7406–7412
17. Gupta, S., Campbell, D., Derijard, B., and Davis, R. J (1995) *Science* **267,** 389–393
18. Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyriakis, J. M., and Zon, L. I. (1994) *Nature* **380,** 75–79
19. Tournier, C., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1997) *Proc. Natl. Acad. Sci.* U. S. A. **84,** 7337–7342
20. Phelan, D. R., Loveland, K. L., Devereux, L., and Dorow, D. S. (1999) *Mol. Reprod. Dev.* **52,** 135–140
21. Schwarzschild, M. A., Cole, R. L., and Hyman, S. E. (1997) *J. Neurosci.* **17,** 3455–3466
22. Eilers, A., Whitfield, J., Banij, C., Rubin, L. L., and Ham, J. (1998) *J. Neurosci.* **18,** 1713–1724
23. 29 DiFiglia, M., Sapp, E., Chase, K. O., Davies, S. P., Bates, G. P., Vonsattel, J. P., and Aronin, N. (1997) *Science* **277,** 1990–1993
24. Scherzinger, E., Lurz, R., Turmaine, M., Mangiarini, L., Hollenbach, B., Hasenbank, R., Bates, G. P., Davies, S. W., Lehrach, H., and Wanker, E. E. (1997) *Cell* **90,** 549–559
25. Saudou, F., Finkbeiner, S., Deys, D., Greenberg, M. E. (1998) *Cell* **95,** 55–66
26. Go, Y. M., Patel, R. P., Maland, M. C., Park, H., Beckman, J. S., Darley-Usmar, V. M., Jo, H. (1999) *Am. J. Physiol.* **277,** H1647–H1653

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# Kainate Receptor Activation Induces Mixed Lineage Kinase-mediated Cellular Signaling Cascades via Post-synaptic Density Protein 95*

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**Kainate receptor glutamate receptor 6 (GluR6) subunit-deficient and c-Jun N-terminal kinase 3 (JNK3)-null mice share similar phenotypes including resistance to kainite-induced epileptic seizures and neuronal toxicity (Yang, D. D., Kuan, C-Y., Whitmarsh, A. J., Rincon, M., Zheng, T. S., Davis, R. J., Rakis, P., and Flavell, R. (1997) *Nature* 389, 865–869; Mulle, C., Seiler, A., Perez-Otano, I., Dickinson-Anson, H., Castillo, P. E., Bureau, I., Maron, C., Gage, F. H., Mann, J. R., Bettler, B., and Heinemmann, S. F. (1998) *Nature* 392, 601–605). This suggests that JNK activation may be involved in GluR6-mediated excitotoxicity. We provide evidence that post-synaptic density protein (PSD-95) links GluR6 to JNK activation by anchoring mixed lineage kinase (MLK) 2 or MLK3, upstream activators of JNKs, to the receptor complex. Association of MLK2 and MLK3 with PSD-95 in HN33 cells and rat brain preparations is dependent upon the SH3 domain of PSD-95, and expression of GluR6 in HN33 cells activated JNKs and induced neuronal apoptosis. Deletion of the PSD-95-binding site of GluR6 reduced both JNK activation and neuronal toxicity. Co-expression of dominant negative MLK2, MLK3, or mitogen-activated kinase kinase (MKK) 4 and MKK7 also significantly attenuated JNK activation and neuronal toxicity mediated by GluR6, and co-expression of PSD-95 with a deficient Src homology 3 domain also inhibited GluR6-induced JNK activation and neuronal toxicity. Our results suggest that PSD-95 plays a critical role in GluR6-mediated JNK activation and excitotoxicity by anchoring MLK to the receptor complex.**

AQ: A

Glutamate, the major excitatory neurotransmitter in the central nervous system, gates three types of ionotropic receptors: NMDA,[1] AMPA, and kainate (3). Five kainate receptor subunits in two homology groups have been identified: KA1, KA2, and GluR5, GluR6, and GluR7 (4, 5). Expression of individual GluR5–7 subunits in heterologous systems results in homomeric receptors that respond to glutamate or kainic acid with a rapidly desensitizing current (5, 6). KA1 and KA2, on the other hand, are functional only when coexpressed with GluR5, -6, or -7 (5, 6).

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PSD-95, also known as synapse-associated protein 90, is a scaffold protein that contains three PDZ domains, a SH3 domain, and a guanylate kinase domain (7). The PDZ domains have been shown to bind to the C terminus of NMDA receptor NR2 and kainate receptor GluR6 subunits, and these interactions are important for the clustering of NMDA or kainate receptors in the postsynaptic membrane (7–9). In addition, PSD-95 also binds to cytoskeletal linker proteins and cytoplasmic signaling proteins such as neuronal nitric oxide synthase and the Src family protein tyrosine kinase Fyn (10, 11). PSD-95 appears to link NMDA or kainate receptors to a variety of cellular signaling cascades. In transgenic mice lacking PSD-95, although the localization of NMDA receptors at post-synaptic density remains unaltered, the frequency dependence of NMDA-dependent long-term potentiation and long-term depression is shifted, and spatial learning is severely impaired (12). Suppression of PSD-95 expression inhibits NMDA receptor-mediated activation of nitric-oxide synthase and neuronal excitotoxicity (13), which suggests that PSD-95 is critical in coupling glutamate receptors to cellular signaling networks and plays an important role in their biological function within the central nervous system.

AQ: B

JNK is a major stress-activated kinase in mammalian systems that is implicated in mediating neuronal death induced by various detrimental stimuli and by glutamate-mediated excitotoxicity (14–16). In the absence of JNK3, a neuronal form of JNK, kainic acid-induced seizure activity and neuronal degeneration are significantly attenuated (1). This phenotype is strikingly similar to that observed in GluR6-deficient mice (2), which suggests that JNK activation may be involved in GluR6-mediated excitotoxicity.

Both MLK2 and MLK3 are members of the mixed lineage kinase family typified by a N-terminal SH3 domain, a middle kinase domain, and a C-terminal proline-rich region that may bind to SH3 domain-containing proteins. MLK2/3 can directly bind and activate MKK4 and MKK7, which in turn phosphorylate and activate JNKs (17–18). Studies from our group and others show that expression of MLK2 induces JNK activation and apoptotic cell death (18–19).

The current study was undertaken to investigate the molec-

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[1] The abbreviations used are: NMDA, *N*-methyl-D-aspartate; GluR6, glutamate receptor 6; MLK, mixed lineage kinase; PSD-95, post-synaptic density protein 95 (also known as SAP-90, synapse-associated protein 90); JNK, c-Jun N-terminal kinase; SH3, Src homology 3; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole proprionate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; MKK, mitogen-activated kinase kinase; HD, Huntington's disease GST, glutathione *S*-transferase; TUNEL, Tdt-mediated dUTP-biotin nick end labeling; KA, kainate acid; MAPK, mitogen-activated protein kinase.

ular mechanism of GluR6-mediated excitotoxicity. We hypothesized that the SH3 domain of PSD-95 binds to the proline-rich region of MLK2 or MLK3 and recruits these kinases to the proximity of GluR6, leading to JNK activation and neuronal apoptosis.

## MATERIALS AND METHODS

*Cell Culture*—Cell culture conditions for HN33 cells, an immortalized rat hippocampal neuronal cell line, and 293T cells have been described previously (20). c-Myc-tagged full-length MLK2 was a generous gift of Dr. A. Hall (University College of London, United Kingdom) and Flag-tagged full-length MLK3 was a gift from Dr. A. Rana (Massachusetts General Hospital, Boston, MA). HN33 or 293T cells at 50 to 60% confluence were washed once with serum-free medium prior to transfection. Transfection was performed using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's instructions. 10–20 $\mu$g of plasmids with 10 $\mu$l of LipofectAMINE/10-cm plate was used in transfection experiments.

*Co-immunoprecipitation and in Vitro Binding Assays*—Antibodies for PSD-95 and GluR6 were purchased from Upstate Biotechnology Inc., and M2, 9E10, and MLK3 antibody were purchased from Santa Cruz. A peptide selected from the MLK2 sequence (amino acids 248–265 DFGLAREWHKTTKMSAAG) was conjugated to KLH for immunization of rabbits, and the resulting polyclonal antibody was purified. The antibody recognizes a 105–110-kDa protein band as expected for MLK2 in the rat brain lysates and the 9E10 immunoprecipitates of 293T cells transfected with c-Myc-tagged MLK2, but is not found in wild-type 293T cell lysates, which lack MLK2. This protein band is no longer detectable when the antibody was pre-absorbed with the peptide antigen (data not shown). Preparation of cell or whole brain lysates and co-immunoprecipitation were conducted as described previously (9, 21). Precipitated proteins were analyzed by Western blotting using an antibody as indicated in the figure legends. A Kodak 440 Image Station was used to analyze and quantify blots. Construction and purification of different PSD-95 GST fusion proteins have been described previously (9). 1–2 $\mu$g of GST fusion protein was used for *in vitro* binding studies.

*JNK and TUNEL Assays*—16 h after transfection, HN33 cells were lysed with 1% Triton X-100 lysis buffer (20). JNK was assayed as described previously (19, 20). 24 to 48 h following transfection, cells were fixed and TUNEL staining was performed as previously described (19, 20). Most apoptotic HN33 cells were detached from the slides and TUNEL staining was performed on the remaining attached cells. TUNEL negative cells (*i.e.* living cells) were counted in the ×20 power field at four different locations, and 600–800 cells were counted in the control.

## RESULTS

To test our hypothesis, we first examined whether MLK2 or MLK3 was associated with PSD-95 in 293T cells coexpressing c-Myc-tagged MLK2 or Flag-tagged MLK3. Wild-type 293T cells which lack PSD-95, served as a negative control. 9E10 or M2 antibodies, which detect the c-Myc or Flag epitopes, respectively, were incubated with 293T cell lysates and immunoprecipitated proteins were analyzed by an anti-PSD-95 monoclonal antibody. As shown in Fig. 1*A*, PSD-95 was detected in 9E10 or M2 immunoprecipitates only from 293T cell lysates co-transfected with MLK2/3 and PSD-95 but was not detected from wild-type cell lysates. Next, we examined whether MLK2 or MLK3 is associated with PSD-95 in HN33 cells or in the rat brain. PSD-95 was detected in MLK2/3 immunoprecipitates from HN33 cell lysates or whole rat brain lysates but not in precipitates obtained with preimmune serum or peptide-antigen preabsorbed MLK2 antibody (Fig. 1*B*). Conversely, immunoprecipitates of PSD-95 also contained MLK2 (Fig. 1*C*) or MLK3 (Fig. 1*D*).

These data indicate that PSD-95 is associated with MLK2/3 in neuronal cells and rat brain tissues. PSD-95 is known to bind to GluR6 (9). Because MLK2/3 is associated with PSD-95, they may be indirectly associated with GluR6 in the brain. As shown in Fig. 1*E*, GluR6 was detected in both MLK2 and MLK3 immunoprecipitates but not precipitates obtained with preimmune serum.

We next explored the molecular basis for the association of PSD-95 with MLK2/3. Both MLK2 and MLK3 contain a proline-rich region that may bind to the SH3 domain of PSD-95. PSD-95 GST fusion proteins containing PDZ1 (amino acids 2–151), PDZ2 (amino acids 156–266), PDZ3 (amino acids 302–401), SH3 (amino acids 402–500), GK (amino acids 512–724), or SH3-GK (SGS, amino acids 402–724) domain were constructed and subsequently purified (9). These GST fusion proteins were incubated with c-Myc-tagged MLK2 or Flag-tagged MLK3 expressed in 293T cells. We found that MLK2 only bound to PSD-95 SH3 or SH3-GK domain (SGS) GST fusion proteins but not to any of PSD-95 PDZ GST fusion proteins or to GST alone (Fig. 2). We also observed that MLK3 bound only to the SH3 domain but not to PDZ or GK domains of PSD-95 (data not shown).

The above data suggest that GluR6, PSD-95, and MLK2/3 may exist in a complex. Because MLK2/3 is a strong activator of JNKs and both JNK and GluR6-deficient mice are resistant to kainic acid-induced seizures and neuronal degeneration (1, 2), we reasoned that GluR6-mediated excitotoxicity might be mediated by the MLK-JNK pathway. Thus, we investigated whether expression of GluR6 induces JNK activation in HN33 cells. Twenty-four hours following transfection, cells were treated with 100 $\mu$M kainic acid for 5 min and JNK activity was analyzed by an anti-phospho-JNK antibody. Expression of vector alone (negative control) did not induce JNK activation, whereas expression of GluR6 caused an elevated JNK activity, which was further increased upon stimulation with 100 $\mu$M kainic acid (Fig. 3*A*). To eliminate the possibility that JNK activation was due to activation of other ionotropic glutamate receptors, we employed a series of receptor antagonists. Addition of 10 $\mu$M CNQX, a kainate and AMPA receptor antagonist, to the transfection medium significantly attenuated JNK activation while addition of 10 $\mu$M GYKI 52466, a selective AMPA receptor antagonist, had no effect. Addition of (−)-AP-5, a selective NMDA receptor antagonist caused a weaker inhibition of GluR6-mediated JNK activation. Coaddition of 10 $\mu$M (−)-AP-5 and CNQX to the medium nearly completely blocked JNK activation (Fig. 3*A*). These data suggest that activation of GluR6 largely accounts for elevated JNK activity in HN33 cells.

Because MLK2/3 is a strong activator of JNK (17, 18), we next examined whether either of these two kinases are involved in GluR6-mediated JNK activation. As shown in Fig. 3*B*, coexpression of dominant negative MLK2 or MLK3 or MKK4 and MKK7 significantly inhibited GluR6-induced JNK activation in HN33 cells. Dominant negative MLK3 appeared to be less effective. This may be due to its lower abundance in HN33 cells. These data suggest that GluR6 may activate MLK2/3-mediated cellular signaling cascades in HN33 cells. We believe that GluR6 anchors and activates MLK2/3 via a complex involving PSD-95. If this hypothesis is correct, then either deletion of the PSD-95-binding site of GluR6 or mutation of the SH3 domain of PSD-95 should disrupt assembly of the GluR6·PSD-95·MLK2/3 complex and block JNK activation mediated by the receptor. As shown in Fig. 3*A*, deletion of the C-terminal four amino acids of GluR6 (GluR6Δ) nearly completely blocked JNK activation mediated by the receptor. Co-expression of PSD-95 with a mutated SH3 domain (W470A) also significantly inhibited GluR6-mediated JNK activation (Fig. 3*B*).

Next, we investigated whether expression of GluR6 induces apoptotic cell death in HN33 cells. Expression of GluR6 induced rapid apoptosis, and TUNEL-positive cells were detectable 24 h following transfection. At 48 h post-transfection, about 30–35% of cells were apoptotic (Fig. 4*A*). Addition of a nonspecific caspase inhibitor, zDEVD-frm, to the transfection medium completely blocked neuronal death (Fig. 4*A*), support-

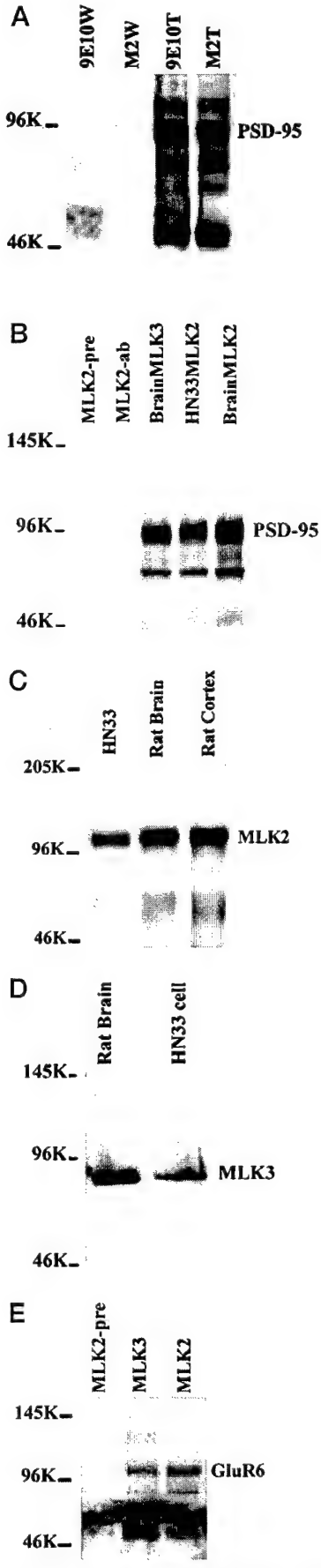


FIG. 1. **Association of PSD-95 with MLK2 or MLK3.** All data presented represent a typical experiment that has been repeated 2–3 times with similar results. *A*, full-length PSD-95 and c-Myc-tagged MLK2 or Flag-tagged MLK3 constructs were transiently expressed in 293T cells. Cell lysates were immunoprecipitated with 9E10 or M2 and the blot was analyzed by an anti-PSD-95 antibody. 9E10W or M2W, immunoprecipitated with wild-type 293T cell lysates; 9E10T or N2T, immunoprecipitated with transfected 293T cell lysates. *B*, detection of PSD-95 in MLK2 or MLK3 immunoprecipitates: *MLK2-pre*, brain lysates immunoprecipitated with MLK2 preimmune serum; *MLK2-ab*, brain lysates immunoprecipitated with peptide-antigen preabsorbed MLK2 antibody; *BrainMLK3*, *HN33MLK2*, or *BrainMLK2*, brain or HN33 cell lysates immunoprecipitated with an anti-MLK3 or anti-MLK2 antibody. *C*, detection of MLK2 in PSD-95 immunoprecipitates: as indicated in the figure, HN33 cell or brain lysates were immunoprecipitated with an anti-PSD-95 antibody and the blot was analyzed by an anti-MLK2 antibody. *D*, detection of MLK3 in PSD-95 immunoprecipitates using HN33 cell or brain lysates. *E*, detection of GluR6 in MLK2 or MLK3 immunoprecipitates but not in the preimmune serum of the MLK2 antibody.

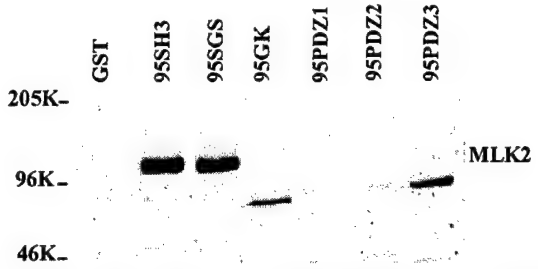


FIG. 2. **The SH3 domain of PSD-95 mediates its binding to MLK2.** c-Myc-tagged MLK2 or Flag-tagged MLK3 expressed in 293T cells were incubated with ~2 μg of GST alone or different PSD-95 GST fusion proteins and the blot was analyzed by an anti-PSD-95 antibody. *95SH3, 95SGS, 95GK, 95PDZ1, 95PDZ2,* or *95PDZ3* indicates the SH3, SH3-GK, GK, PDZ1, -2, or -3 domain GST fusion proteins. The data represent a typical experiment that has been repeated three times with similar results.

ing that cell death is apoptotic. PSD-95 has been implicated in activation of nNOS by NMDA receptors (13). To test the role of nNOS in GluR6-mediated neuronal toxicity in HN33 cells, $N^{\omega}$-propylarginine, a selective nNOS inhibitor, was added to the transfection medium. As shown in Fig. 4*A*, the nNOS inhibitor had almost no effect on cell death, suggesting that nNOS does not contribute to GluR6-mediated neuronal toxicity in HN33 cells. Addition of 500 μM kainic acid to the medium 24 h following transfection significantly enhanced neuronal toxicity: the number of apoptotic cells was increased to 60% at 48 h post-transfection, compared with 40% without kainic acid (Fig. 4*B*). Addition of 10 μM CNQX significantly attenuated neuronal toxicity: over 80% of cells survived (Fig. 4*B*). Consistent with the data from JNK activation, GYKI 52466 had no effect while coaddition of 10 μM (−)-AP5 with CNQX nearly completely blocked neuronal toxicity (Fig. 4*B*). These data indicate that GluR6 plays a major role in the induction of neuronal toxicity in HN33 cells.

Next, we investigated whether activation of MLK2/3-mediated signaling cascades is involved in GluR6-mediated neuronal toxicity in HN33 cells. As shown in Fig. 4*C*, expression of a mutated GluR6 that does not bind to PSD-95 did not cause significant cell death, and addition of kainic acid did not enhance the toxicity. Co-expression of dominant negative MLK2 or MKK4 and MKK7 significantly inhibited apoptosis induced by GluR6 (Fig. 4*D*), suggesting that MLK2-mediated signaling cascades do play a significant role in the induction of neuronal toxicity. Co-expression of dominant negative MLK3 had a smaller inhibitory effect (data not shown) while co-expression of PSD-95 with a deficient SH3 domain inhibited GluR6-mediated apoptotic cell death, although the extent of the inhibition was much weaker than that mediated by dominant negative MLK2 or MKK4 and MKK7 (Fig. 4*D*). These data indicate that

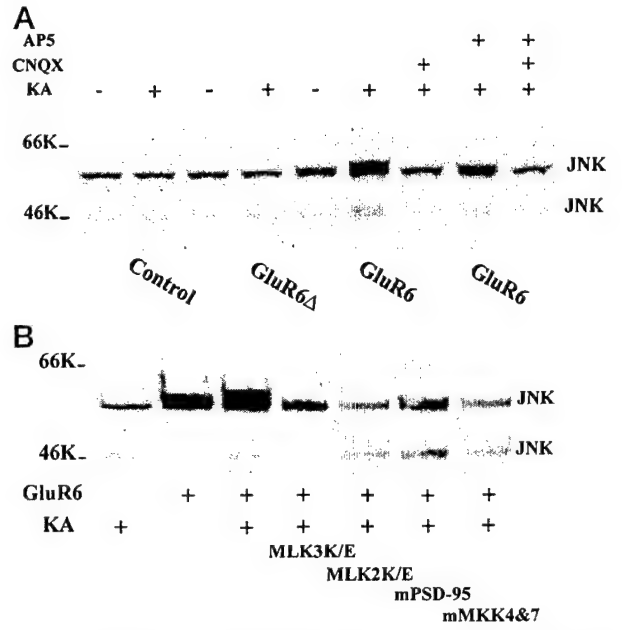


FIG. 3. **Activation of JNK by GluR6.** 20 h following transfection, wild-type or GluR6 transfected HN33 cell lysates were analyzed for JNK activity using an anti-phospho-JNK antibody. The data is from a typical experiment that has been repeated at least three times. *A,* activation of GluR6 accounts for JNK activation in HN33 cells. Different receptor antagonists as indicated in the figure were added to the transfection medium and 20 h post-transfection, cells were stimulated with 100 μM kainic acid for 5 min followed by analysis of JNK activity. GluR6Δ, GluR6 with deleted C-terminal four amino acids. *B,* dominant negative MLK2/3 or MKK4 and MKK7 or SH3 domain-deficient PSD-95 significantly inhibits JNK activation induced by GluR6. *MLK2K/E, MLK3K/E,* or *mMKK4/7,* dominant negative form of kinases; *mPSD-95,* PSD-95 (W470A).

GluR6 activates MLK2/3-mediated cellular signaling cascades via PSD-95 to mediate apoptosis in HN33 cells.

## DISCUSSION

GluR6 activation induces neuronal degeneration in hippocampus (2, 22), and GluR6-deficient mice exhibit a resistance to neuronal degeneration and seizure induced by kainic acid (2). A similar phenotype was also observed in JNK3 knockout mice (1), suggesting that activation of JNKs may be involved in excitotoxicity mediated by GluR6. Although some reports have shown that stimulation of kainate receptors mediates JNK activation (23), it is unclear how GluR6 mediates JNK activation and whether inhibition of JNK activation blocks GluR6-mediated neuronal toxicity. In this study, we provide evidence that GluR6 activates JNKs via the PSD-95-MLK2/3-signaling pathway. Expression and activation of GluR6 causes JNK activation and apoptosis in HN33 cells. Co-expression of dominant negative MLK2 or MLK3 significantly inhibits both JNK activation and neuronal toxicity induced by the receptor. Additionally, co-expression of dominant negative MKK4 and MKK7, which are immediate upstream activators of JNKs, also significantly inhibited GluR6-mediated JNK activation and apoptosis. MLK2 or MLK3 is associated with PSD-95 and GluR6 in intact neuronal cells and in rat brain tissues, suggesting that MLK2 or MLK3 may assemble into a signaling complex with PSD-95 and GluR6. Deletion of the PSD-95-binding site of GluR6 significantly abolished the ability of the receptor to induce JNK activation and apoptosis in HN33 cells, suggesting that binding of PSD-95 to the receptor is necessary for neuronal toxicity to occur. The SH3 domain of PSD-95 mediates its binding to MLK2/3; and mutation of the SH3 domain of PSD-95 significantly attenuates both JNK activation and apoptosis

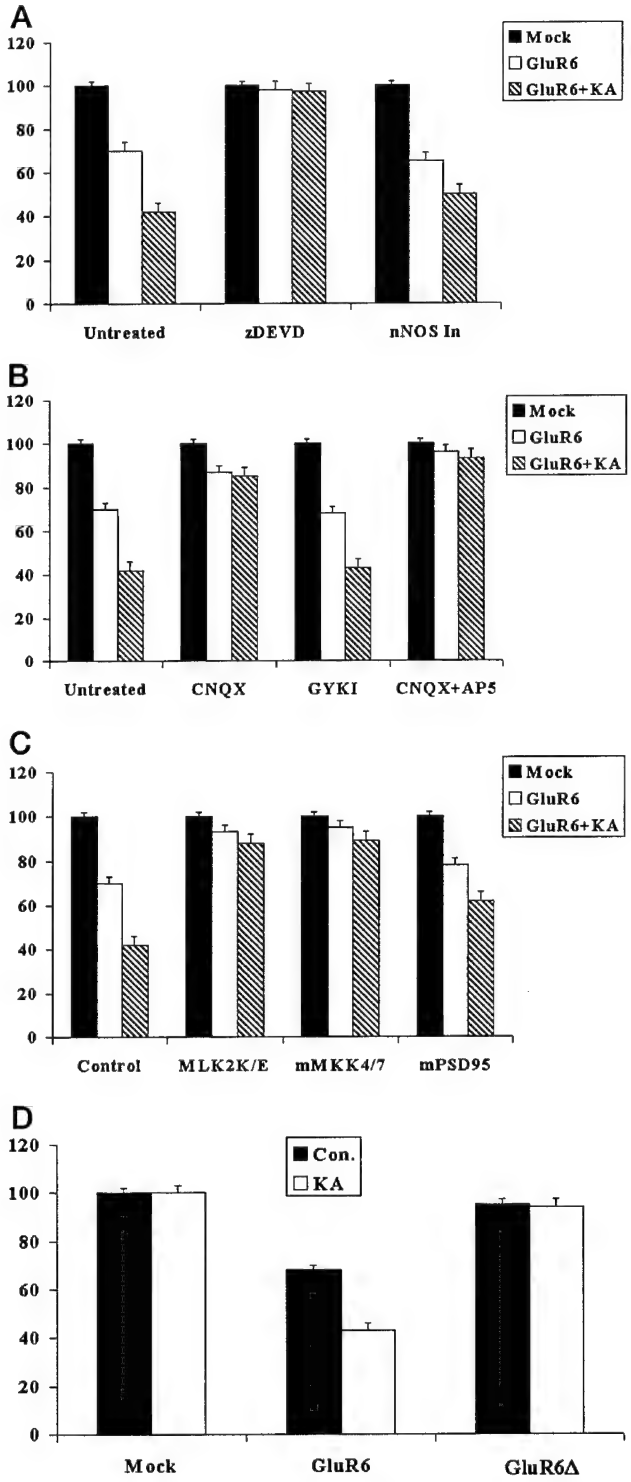


FIG. 4. **GluR6 mediates apoptotic cell death in HN33 cells.** HN33 cells were transfected with vector alone (*mock*) or different plasmids as indicated in the figures. 48 h post-transfection, cells were fixed and TUNEL staining was carried out and TUNEL-negative cells were counted. The data are the average of three independent experiments. *Con,* control. *A,* GluR6-mediated apoptosis was blocked by a nonspecific caspase inhibitor zDEVD-frm but not by a selective nNOS inhibitor $N^{\omega}$-propyl-L-arginine. *B,* activation of GluR6 mostly accounts for apoptosis. Individual receptor antagonist was added into the transfection medium and 500 μM kainic acid was added into the medium 24 h post-transfection. *C,* HN33 cells were co-transfected with dominant negative form of MLK2 (MLK2K/E) or MKK4 and -7 (mMKK4/7) or PSD-95W470A (mPSD-95) as indicated in the figure followed by TUNEL assay. *D,* deletion of the C-terminal four amino acids of GluR6 (GluR6Δ) inhibits its ability to induce apoptosis in HN33 cells.

mediated by GluR6, suggesting that binding of MLK2/3 to the SH3 domain of PSD-95 is critical for induction of neuronal toxicity. MLK2/3 is a constitutively active kinase (17, 18). Our explanation is that assembly of the GluR6·PSD-95·MLK2/3 complex occurs upon activation of the receptor and promotes exposure of the kinase domain to ATP and activation of the downstream signaling pathway.

PSD-95 has been implicated in excitotoxicity and the induction of activation of nNOS by NMDA receptors (13). In HN33 cells, nNOS activation does not contribute to GluR6-induced neuronal toxicity because the nNOS inhibitor has no effect on apoptosis induced by the receptor. Some reports suggest that p38 MAPK is involved in the neuronal toxicity mediated by NMDA receptors (24). We found that addition of SB 203580, a selective p38 MAPK inhibitor, to the transfection medium had no any effect on GluR6-induced neuronal death. In addition, GluR6 failed to activate MAPK in HN33 cells. These data are consistent with the fact that, at moderate expression levels, MLK2/3 selectively activates the MKK4/7-JNK pathway (17, 18).

Kainate receptor-mediated excitotoxicity may play an essential role in the pathogenesis of intra-striatal injection of kainic acid in rats creates HD-like pathology (25). The number of kainate receptors is significantly reduced and high affinity receptors are almost absent in the brains of HD patients and HD-transgenic mice (26, 27). Moreover, the TAA repeat polymorphism of the *GluR6* gene has been linked to a younger onset of HD (28, 29). In a previous report, we showed that activation of MLK2-mediated signaling cascades may contribute to neuronal death induced by polyglutamine-expanded huntingtin. The current study suggests that GluR6 activation and mutated huntingtin may share a similar molecular mechanism to induce neuronal death. This would help to explain the potential role of the receptor in the pathogenesis of HD. In summary, our present study gives new insight into how PSD-95 links a kainate receptor to stress-activated kinase signaling cascades.

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REFERENCES

1. Yang, D. D., Kuan, C.-Y., Whitmarsh, A. J., Rincon, M., Zheng, T. S., Davis, R. J., Rakis, P., and Flavell, R. (1997) *Nature* **389,** 865–869
2. Mulle, C., Seiler, A., Perez-Otano, I., Dickinson-Anson, H., Castillo, P. E., Bureau, I., Maron, C., Gage, F. H., Mann, J. R., Bettler, B., Heinemmann, S. F. (1998) *Nature* **392,** 601–605
3. Hollman, M., and Heinemann, S. (1994) *Annu. Rev. Neurosci.* **17,** 31–108
4. Chittajallu, R., Braithwaite, S. P., Clarke, V. R. J., and Henley, J. M. (2000) *Trends Pharmacol. Sci.* **20,** 26–35
5. Frerking, M., and Nicoll, R. A. (2000) *Curr. Opin. Neurobiol.* **10,** 342–351
6. Kornau, H.-C., Schenker, L. T., Kennedy, M. B., and Seeberg, P. H. (1995) *Science* **269,** 1737–1740
7. Kim, E., Cho, K. O., Rothschild, A., and Sheng, M. (1996) *Neuron* **17,** 103–113
8. Niethammer, M., Kim, E., and Sheng, M. (1997) *Neuron* **16,** 2157–2163
9. Garcia, E. P., Metha, S., Blair, L. A., Wells, D. G., Shang, J., Fukushima, T., Fallon, J. R., Garmer, C. C., and Marshall, J. (1998) *Neuron* **21,** 727–736
10. Brenman,, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santillano, D. R., Wu, Z., Huang, F., Xia, H., Peters, M. F., Froehner, S. C., and Bredt, D. S. (1996) *Cell* **84,** 757–767
11. Tezuka, T., Umemori, H., Akiyama, T., Nakanishi, S., and Yamamoto, T. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 435–440
12. Migaud, M., Charlesworth, P., Dempater, M., Webster, L., Watabe, A. M., Makhinson, M., He, Y., Ramsay, M. F., Morris, R. M., Morrison, J. H., O'Dell, T. J., and Grant, S. G. N. (1998) *Nature* **396,** 433–439
13. Sattler, R., Xiong, Z., Lu, W.-Y., Hafner, M., MacDonald, J. F., and Tymianski, M. (1999) *Science* **284,** 1845–1848
14. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M., Avruch, J., and Woodgett, R. J. (1994) *Nature* **369,** 156–160
15. Derijard, B., Raingeaud, J., Barrett, T., Wu, I., H, Han, J., Ulevitch, R. J., and Davis, R. J. (1994) *Science* **267,** 682–685
16. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995) *Science* **270,** 1326–1339
17. Hirai, S., Katoh, M., Terada, M., Kyriakis, J. M., Zon, L. I., Rana, A., Avruch, J., and Ohno, S. (1997) *J. Biol. Chem.* **272,** 15167–15173
18. Nagata, K., Puls, A., Futter, C., Aspenstrom, P., Schaefer, E., Nakata, T., Hirokawa, N., and Hall, A. (1998) *EMBO J.* **17,** 149–158
19. Liu, Y. F., Dorow, D., and Marshall, J. (2000) *J. Biol. Chem.* **275,** 19035–19040
20. Liu, Y. F. (1998) *J. Biol. Chem.* **273,** 28873–28877
21. Liu, Y. F., Deth, C. R., and Devys, D. (1997) *J. Biol. Chem.* **272,** 8121–8124
22. Telfeian, A. E., Federoff, H. J., Leone, P., During, M. J., and Williamson, A. (2000) *Neurobiol. Dis.* **7,** 362–374
23. Schauwecker, P. E. (2000) *Brain Res.* **884,** 116–128
24. Kawasaki, H., Morooka, T., Shimohama, S., Kimura, J., Hirano, T., Gotoh, Y., and Nishida, E. (1997) *J. Biol. Chem.* **272,** 18518–18521
25. Beal, M. F., Kowall, N. W., Ellison, D. W., Mazurek, M. F., Swartz, K. J., and Martin, J. B. (1986) *Nature* **321,** 168–171
26. Wagster, M. V., Hedreeen, J. C., Peyser, C. E., Folstein, S. E., and Ross, C. A. (1994) *Exp. Neurol.* **127,** 70–75
27. Cha, J. H., Frey, A. S., Alsdorf, S. A., Kerner, J. A., Kosinski, C. M., Mangiarini, L., Penney, J. B. J.r, Davies, S. W., Bates, G. P., and Young, A. B. (1999) *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **354,** 981–989
28. MacDonald, M. E., Vonsattel, J. P., Shrinidhi, J., Couropmitree, N. N., Cupples, L. A., Bird, E. D., Gusella, J. F., and Myers, R. H. (2000) *Neurology* **12,** 1330–1332
29. Rubinsztein, D. C., Leggo, J., Chiano, M., Dodge, A., Norbury, G., Rosser, E., and Craufurd, D. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3872–3876

POSTER

## Activation of MLK is involved in neuronal toxicity induced by GluR6 receptors

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Introduction of kainic acid into rat hippocampus causes rapid neuronal apoptosis while knockout of JNK3, a neuronal form JNKs leads to hippocampal neurons of transgenic mice to resist neuronal excitotoxicity induced by kainic acid. To date, how activation of kainate receptors induces JNK activation remains unknown. Our hypothesis is that MLK2/3, the upstream activators of JNKs may bind to PSD-95, a synaptic proteins that binds to the C-terminus of GluR6 receptors and involved in JNK activation and neuronal toxicity mediated by the receptors. By co-immunoprecipitation, we observed that MLK2 or MLK3 is associated with PSD-95. *In vitro* binding studies showed that the SH3 domain of PSD-95 mediates its binding to MLK2 or MLK3. Transfection of GluR6 receptors in hippocampal neuronal cells induced JNK activation and neuronal apoptosis, while transfection of GluR6 receptors with a deletion of the PSD-95 binding motif failed to mediated JNK activation and neuronal death. Co-transfection of kinase dead of MLK2 significantly attenuated neuronal toxicity induced by GluR6 receptors. In summary, our studies suggested that activation of MLK may be involved in JNK activation and neuronal toxicity mediated by GluR6 receptors. (This project is supported by US Army Medical Research and Materiel Command). **Key words: GluR6 receptor, MLK, JNK.**

**POSSIBLE INVOLVEMENT OF P53 IN THE PATHOLOGY OF HUNTINGTON'S DISEASE.** A. Sawa*, S. Igarashi, P.H. Hwang, Y. Taya, M. Fujimuro, X. Luo, C.A. Ross, S.H. Snyder. *Dept. of Neuroscience, Johns Hopkins Univ School of Med., Baltimore, MD, USA*

Cellular and animal models suggest that nuclear and mitochondrial dysfunction is crucial to HD pathogenesis. p53 is a transcription factor which regulates cell cycle and apoptosis. After various cellular stimuli such as DNA damage and binding to HIF-1, p53 is known to accumulate and become activated in the nucleus. Recently p53 has been reported to interact with Huntingtin (Htt) directly. We have evaluated p53 accumulation after the expression of Htt. In differentiated PC12 cells stably expressing an inducible Htt construct, and in transient transfection of Htt constructs into N2a cells, HEK293 cells, and Hela cells, we observed an accumulation of p53 in the nuclear fraction only upon mutant Htt, but not normal Htt. The accumulation occurs prior to robust cell death. Previous studies have shown that posttranslational modifications such as phosphorylation and acetylation play a role in p53 accumulation. Phophorylation on Ser-15 and -20 is important for the accumulation after DNA damage. We did not see a robust change in phosphorylation of Ser-15 and -20 after Htt expression, but are testing other sites using phospho-specific antibodies. Our data, while preliminary, are consistent with a possible role for p53 in HD pathogenesis. *Supported by: USPHS grant MH-18501.*

**NEURONAL DEATH AND AGGREGATE FORMATION IN NONHUMAN PRIMATES FOLLOWING INTRASTRIATAL VIRAL EXPRESSION OF POLYGLUTAMINE TRACTS.** J.H Kordower[1*], S.- Suhr[2], L.- Leventhal[1], S.- Palfi[1], M.- Emborg[1], B. Kaspar[2], F.H.- Gage[2], M.-C. Senut[2]. [1]*Dept Neurological Sciences, Rush Presbyterian Med Ctr, Chicago, IL, USA;* [2]*Laboratory of Genetics, Salk Institute, San Diego, CA, USA*

Eight inherited diseases including Huntington's disease (HD) have a genetic basis that is linked to the expanded polyglutamine (CAG) repeats. Transgenic rodents expressing expanded CAG repeats have recently been established. However, the characterization of primate models in which neuronal death can occur in a site specific manner via mechanisms identical to that seen in human disease may be a critical step in understanding disease pathogenesis and evaluating novel experimental treatment approaches. In this regard, six Cebus monkeys received MRI guided injections bilaterally into the caudate nucleus (n=2) and putamen (n=3) of an adeno-associated virus (AAV) encoding for normal (13polyQ) or pathologic (97polyQ) CAG repeats lengths. Monkeys were sacrificed 1-6 weeks following AAV injection. Transgene expression was identified in vivo via GFP fluorescence. Injections of control 13 polyQ-AAV failed to induce aggregate formation or neuronal death within the striatum. In contrast, striata receiving 97 polyQ-AAV displayed ubiquinated neuronal aggregates and a loss of neurons as seen in Nissl, NeuN, and calbindin stains. While further studies are needed to confirm the specificity of these findings, thepresent data suggest that the viral expression of expanded polyglutamine tracts is toxic to striatal neurons in nonhuman primates and may serve as an animal model of genetic diseases such as HD. *Supported by: NS35078.*

## 203.7

**HUNTINGTIN INTERACTS WITH SYNAPTIC ASSOCIATED PROTEINS.** Y. Liu*, Y. Sun. *Pharmaceutical Sci, Northeastern Univ, Boston, MA, USA*

Huntingtin Interacts with Synaptic Associated Proteins Ying Sun and Ya Fang Liu* Dept. of Pharmacology, Northeastern Univ. Boston MA 02115 Normal huntingtin is richly expressed in never terminals and associated with synaptic vesicles. The current study is undertaken to investigate the potential interaction of normal huntingtin and synaptic associated proteins SAP-90 and SAP-97. We found that normal huntingtin is associated with both SAP90 and SAP-97 in 293T cells and in human brain cortex. In vitro binding studies show the SH3 domain of SAP-90 or SAP-97 mediates their interaction with normal huntingtin. The polyglutamine expansion in huntingtin inhibits its ability to bind to the SH3 domain of SAP-90 and SAP97. Because SAP-90 and SAP-97 are known to direct bind and regulate the activity of NMDA or kainite receptors, our studies suggest that normal huntingtin may be a modulator of glutamate receptor-operated channels while this function may be alter upon the polyglutamine expansion of the protein in Huntington's disease.

## 203.8

**MOUSE MODEL FOR MACHADO JOSEPH DISEASE WITH CLEAVAGE OF MUTANT ATAXIN-3.** V. Colomer[1*], S.M. Katzen[1], J. Mez[1], N. Kurtis[1], M.W. Becher[4], Y. Trottier[5], H. Paulson[6], D.R. Borchelt[3], A. Kakizuka[7], A.H. Sharp[1], G. Schilling[3], C.A. Ross[2]. [1]*Div. of Neurobiology Dept. of Psychiatry Meyer bldg,* [2]*Div. of Neurobiology Dept. of Psychiatry Ross bldg,* [3]*Dept. of Pathology, Johns Hopkins Univ. School of Medicine, Baltimore, MD, USA;* [4]*Dept. of Pathology, Univ. of New Mexico; Albuquerque, NM, USA,* [5]*IGBMC, CU de Strasbourg, Strasbourg, France,* [6]*Dept. of Neurology, Univ. of Iowa College of Medicine, Iowa City, IA, USA,* [7]*The Fourth Dept., Osaka Bioscience Institute, Osaka, Japan*

Machado Joseph disease (MJD) or spinocerebellar ataxia type 3 (SCA3) is an autosomal dominant cerebellar ataxia caused by an expansion of glutamines in ataxin-3 from 14-40 to 55-80. We generated transgenic mice expressing human ataxin-3 with 20 or 71 glutamines (Ax3Q20 or Ax3Q71) under the control of the mouse prion promoter. Ax3Q71 transgenic mice have an MJD-like phenotype including movement coordination and gait problems progressing to an early death. Neuropathological examination revealed nuclear inclusions stained with H&E or antibodies to ataxin-3. Ax3Q20 transgenic mice are normal. Ax3Q71 transgenic mice do not breed. We are generating them by breeding homozygotes or double transgenics using heterozygous mice expressing mutant ataxin-3 at lower levels. In MJD human or transgenic mouse tissue, we identified a mutant ataxin-3 form smaller than the full length protein. It accumulates in the nucleus of cells throughout the brain. Based on epitope mapping data, we suggest that it is a c-terminal fragment and the cleavage site is within a 19 amino acid region. In summary, we have developed a mouse model for MJD and propose that proteolytic cleavage of ataxin-3 may be involved in pathogenesis. *Supported by: AHA, Gift fund, Ataxia MJD Research Proj. Inc., NIH.*

## 203.9

**A MICROPLATE ASSAY FOR POLYGLUTAMINE AGGREGATION.** V.M. Berthelier, S. Chen, J.B. Hamilton, R.B. Wetzel*. *Department of Medicine, Univ. Tenn. Med. Ctr, Knoxville, TN, USA*

Polyglutamine (polyGln) aggregates are neuropathological markers of expanded CAG repeat disorders like Huntington's disease (HD), but their role in the disease process is unclear. Several studies have found no correlation between aggregate formation and cytotoxicity, whereas others have drawn a close parallel. One possible explanation for this inconsistent data is that the aggregates of importance are 'microaggregates' too small to be detected by available methods. We have established a highly sensitive, fast, reproducible and specific assay capable of detecting very small quantities of polyGln aggregates by virtue of their ability to recruit additional polyGln peptides. PolyGln aggregates made from chemically synthesized peptide are used to coat a 96-well plate and incubated for different times with low concentrations of biotinylated polyGln peptide. Europium-streptavidin addition and time-resolved fluorescence allows us to calculate the rate (fmoles/hr) of incorporation of polyGln peptides into polyGln aggregates. Under optimal conditions we are able to detect as little as 40 pg (0.4 ng/ml) of synthetic polyGln aggregates. In fact, aggregates of recombinant ataxin-3 also produce a signal when bound to microplate wells. This assay is highly specific: biotinylated polyGln peptide exhibits no measurable binding to amyloid A-beta fibrils and vice-versa. Our results suggest that this microplate assay will be a useful test for detection of micropolyGln aggregates in biological materials, in order to study the relationship of polyGln aggregation to HD pathogenesis. The assay may also be valuable in screening for and characterizing anti-aggregation inhibitors. *Supported by: Hereditary Disease Foundation.*

## 203.10

**HSP70 AND HSP40 CHAPERONES SUPPRESS HUNTINGTIN FIBRILLIZATION.** P.J. Muchowski[1,2*], G. Schaffar[2], A. Sittler[3], E.E. Wanker[3], M. Hayer-Hartl[2], F.U. Hartl[2]. [1]*Dept Genetics & Med, Univ Washington, Seattle, WA, USA,* [2]*Cellular Biochemistry, Max-Planck-Institut für Biochemie, Martinsried, Germany,* [3]*Molecular Genetics, Max-Planck-Institut für Molekulare Genetik, Berlin, Germany*

The deposition of amyloid-like protein aggregates in neurons is a hallmark of many neurodegenerative diseases, including those caused by polyglutamine proteins. We characterized the effects of chaperones on the aggregation of huntingtin containing expanded polyglutamine tracts. Only chaperones of the Hsp70 and Hsp40 families influenced huntingtin aggregation. In vitro, Hsp70 and its co-chaperone Hsp40 suppressed the assembly of huntingtin into detergent insoluble amyloid-like fibrils in an ATP-dependent manner and caused the formation of amorphous, detergent soluble aggregates. The chaperones were most active in preventing fibrillization when added during the lag phase of the polymerization reaction. Similarly, increased expression of Hsp70 and Hsp40 in yeast and mammalian cells inhibited the formation of large, peri-nuclear, detergent insoluble huntingtin aggregates. Instead, the accumulation of smaller, chaperone-associated inclusions which were detergent soluble and distributed throughout the cytoplasm were observed. These results suggest that the recently established abilities of Hsp70 and Hsp40 to mitigate polyglutamine-induced neurotoxicity in vivo is based on the potency of Hsp70 and Hsp40 to shift the self association pathway of polyglutamine proteins from amyloidogenic to amorphous aggregation. The results suggest that upregulation of Hsp70 and Hsp40 in neurons of affected patients may represent a viable approach to combating polyglutamine disease. *Supported by: Hereditary Disease Foundation (PJM).*

# CURRICULUM VITAE


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## EDUCATION:

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2. 1985 **M.S.** (Pharmacology), Henan Medical University, P.R.China
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## PROFESSIONAL EXPERIENCE:

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| 1987-1988 | Postdoctoral fellow, Department of Psychiatry, New York University Medical Center. |
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| 1. 1988 | McGill University Studentship |
| 2. 1989-1993 | Medical Research Council of Canada Fellowship |
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| 1989- | Society of Neuroscience |
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1992 Invited speaker, 7th International Catecholamine Symposium
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## PUBLICATIONS:

### Original Articles:


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2.Ofri, D., **Liu, Y.F.,** Ritter, A.M., Hiller, J.M., Simon, E.J. (1992) Characterization of CHAPS solubilized opiate receptors-guanylyl-nucleotide reconstitution agents binding. *J. Neurochem.* **58**,628-635.

3. **Liu, Y.F**. and Quirion, R. (1991) Presence of various carbohydrate moieties including-galactose and N-accetylglucasamine residues on solubilized porcine brain neurokinin 1/substance P receptors. *J. Neurochem.* **57**,1944-1957.

4. **Liu, Y.F**. and Quirion, R. (1992) Modulatory role of glutathione on μ opioid, substance P/neurokinin 1 and kainic acid receptor binding sites. *J. Neurochem.* **59**,1024-1032.

5. **Liu,Y.F**. and Albert, P.R. (1991) Cell-specific signalling of the 5-HT1A receptor: Modulation by protein kinase C and A. J. Biol. Chem.**266**. 23685-23697.

6. **Liu,Y.F**. and Albert, P.R. (1992) Multiple signals mediated by the dopamine-D1 receptor: Involvement of protein kinase A. *Mol. Endo.* **6**,1815-1824.

7. **Liu,Y.F**. Civelli, O., Grandy, D. and Albert, P.R. (1992) Different sensitivity of the long and short forms of the dopamine D2 receptor to protein kinase A. *J. Neurochem.* **59**,311-2317.

8. **Liu, Y.F**. and Albert, P.R. (1992) Receptor-specific coupling of G protein to inhibition of cAMP accumulatiom. *Quan Biol* **17**, 63.

9. Goetzl, E.J., Kishiyamy, J.L., Shames, R.S., **Liu, Y.F.**, Albert, P.R., An, S.-Z., Birk, F.W., Yang, J.-h., Sreedharan, S.P.(1993) Specific inhibition of receptor-dependent human cellular responses by antisense messenger RNA of individual G proteins. *Trans. Assoc. Am. Physicians*. **8**,12-18.

10. Goetzl, E.J., Shames, R.S., Yang, J., Birk, F.W., **Liu, Y.F.**, Albert, P.R., An, S.-Z. (1994) Inhibition of leukocyte response to chemotactic factors by antisense messenger RNA depletion of G proteins. *J. Biol. Chem.* **269**, 809-812.

11. **Liu, Y.F.**, Jakobs, K.H., Rasenick, M.M., and Albert, P.R. (1994) G protein specificity in receptor-effector coupling: analysis of the role of Go and Gi2 in GH4C1 pituitary cells. *J. Biol. Chem.* **269**, 13880-13886.

12. **Liu, Y.F.,** Deth,R.and Devys, D. (1997) SH3 domain-dependent association of huntingtin with EGF receptor signaling complexes. *J. Bio. Chem.* **272**, 8121-8124.

13. **Liu, Y.F**. (1998) Expression of polyglutamine-expanded huntingtin activates the SEK1-JNK pathway and induces apoptosis of hippocampal neuronal cells. *J. Bio. Chem* **273**, 28873-28877.

14. **Liu, Y.F.**, Ghahremani, M.H. Rasenick, M.M., Jakobs, K.H., and Albert, P.R. (1999) Stimulation of cAMP synthesis by Gi-coupled receptors upon of distinct Gαi gene

expression Gi subtype specificity of the 5-HT1A receptor. *J. Biol. Chem.* **274,** 16444-16450

15. **Liu Y.**F. Dorow, D. and Marshall, J. (2000) Activation of MLK2-mediated signaling cascades by polyglutamine-expanded huntingtin. . *J. Biol. Chem.* **275,** 16444-16450.
16. Savinainen, A., Garcia, E., Dorow, D., Marshall, J. and **Liu, Y.F**. (2001) kainate receptor activation induces MLK-mediated cellular signaling cascades via post-synaptic density protein 95. (JBC, in press).
17. David R Phelan, Gareth Price, Ya Fang Liu, and Donna S Dorow Activated JNK Phosphorylates the C-terminal Domain of MLK2 that is Required for MLK2 Induced Apoptosis. *J. Biol. Chem.*
18. Song, C., Shama, A. and Deth, C. R., and **Liu, Y.F.,** (2001) Association of the Dopamine D4 receptor with SH3 domain-containing proteins (JPET, in press).
19. Sun, Y. Reddy, H., Savinainen, A., Song, C. and **Liu, Y.F**. (2001) Polyglutamine-expanded huntingtin promotes sensitization of NMDA receptors via PSD-95 (manuscript in ppraparation).
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